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PRINCIPAL INVESTIGATOR: John W. Harmon, M.D., F.A.C.S.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine
Baltimore, MD 21205

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John W. Harmon, M.D., F.A.C.S.

E-Mail: jharmon@jhmi.edu

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14. ABSTRACT

Wound healing is a controlled coordinated response to tissue injury leading to scar tissue formation. It involves complex interactions between cell types, matrix and soluble mediators. When one or more of these components is disrupted, wound healing is delayed or even halted. There has been some success with topical application of growth factors to improve healing in the face of sepsis and infection. However the effects have been modest. A major limitation of topical application of growth factors is that they are destroyed rapidly by tissue proteases. The gene therapy approach we propose will have the advantage of continually producing growth factors within the wound to constantly replenish tissue levels. Our approach also allows the production of multiple growth factors to maximize potential benefit.

15. SUBJECT TERMS
Military surgery, electroporation, growth factors, gene therapy

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Introduction

Impaired healing of war wounds remains an unsolved military medical problem. It involves complex interactions between cell types, matrix and soluble mediators. In sepsis which is often associated with war wounds, one or more of these components is disrupted. One of the key elements in wound repair is the release of growth factors (e.g. EGF, PDGF, FGF-1, VEGF, KGF-1), which bind to specific receptors on the target cell and activate signal transduction pathways that finally result in proliferation, migration or synthesis of structural elements.
There has been some success with topical application of growth factors to improve healing in the face of sepsis and infection (1-3). However the effects have been modest. A major limitation of topical application of growth factors is that they are destroyed rapidly by tissue proteases. The gene therapy approach we propose will have the advantage of continually producing growth factors within the wound to constantly replenish tissue levels. We are testing this approach using electroporation (EP) to deliver growth factor plasmid DNA to a rodent war wound model.

Body

Describe the research accomplishments associated with each task outlined in the approved Statement of Work.

1. Refined animal model of sepsis.

The originally proposed method of inducing sepsis using E. Coli-impregnated agar pellets to model a war wound in the rat resulted in a high mortality. We identified a suitable model with a mortality rate of less than 10% and a profound effect on wound healing.
The useful model is complete cecal ligation with feces in the cecum. This preparation produces significant, but survivable peritonitis, which would be a common clinical scenario for a military casualty who sustained an abdominal injury.

2. Optimized EP parameters and dosage regimens.

We assessed the ability of *in vivo* electroporation to enhance cutaneous transfection of naked plasmid DNA. A luciferase encoding plasmid driven by a CMV promoter was injected at the wound border. Following plasmid administration, electroporative pulses were applied to injection sites. Luciferase expression in animals was analyzed using an *in vivo* luciferase imaging system.

Pulse parameters were varied over arrange of voltage, duration, and number. The electroporative effect was most marked at a plasmid dose of 50 μg, where an approximate 10-fold increase was seen. Six 100 μs duration pulses of 1750V/cm were found to be the most effective in increasing luciferase activity (Figure 7A of Byrnes et al). Higher voltages than 1800 V/cm tended to either cause arcing of the electric pulse between the electrodes or left some signs of an electrical burn on the animal's skin. Double EP (two applications of EP-assisted transfection separated by 24 hours) did not produce significantly different luciferase expression when compared to single EP (Figure 4 of Lin et al).

We then compared the luciferase plasmid transfection with and without EP using the optimized EP regimen. EP effectively increased expression of luciferase plasmid up to 53-fold compared to vector without EP on day 5 ($p<0.001$, Figure 2 and 3 of Lin et al). The increased activity was maintained for an interval of three weeks, which is appropriate for treatment of wound healing (Figure 4 of Byrnes et al). In addition, transfection was predominately localized to the epithelium, and did not extend into the musculature of the carcass, when plasmid injection and EP were applied to the skin with skin specific parameters (Figure 6 of Lin et al).

3. Demonstrated improvement in wound healing with EP-assisted keratinocyte growth factor-1 (KGF-1) transfection

We measured both the wound areas and wound breaking strength at day 7 in animals with and without the administration of the most effective electroporation settings (6 × 100 μs, 1750 V/cm). EP showed no detrimental effect on these healing parameters. In fact, there was a slight non-significant tendency for the electroporated wounds to have improved healing as evidenced by a smaller open area and greater tensile strength (Figure 9A and B of Byrnes et al).

We then assessed the ability of the PCDNA3.1/KGF-1 expression vector to improve cutaneous wound healing in the septic rat model. The rate of wound healing was impaired by partial cecal ligation to induce sepsis. Average wound area was 4.7 times larger on day 9 in septic rats compared to control ($p<0.001$, Figure 1 of Lin et al). The rate of wound healing improved with KGF-1 expression vector and EP. Wounds were 60% smaller on day 12 versus vector without EP ($p<0.009$, Figure 7 of Lin et al). In addition, the quality of healing was assessed histologically using a point scale from 1-4 (Figure 8 of Lin et al). The quality of healing using KGF-1 vector with EP was scored 3.0 +/- 0.3 and was significantly better than that of 1.8 +/- 0.3 for treatment with vector alone ($p<0.05$, Figure 9 of Lin et al).

With the positive effect on wound quality noted histologically, we did not perform collagen assays.

Disappointingly, multiple growth factors do not improve wound closure as compared to KGF-1 alone.

Key Research Accomplishments

- E. coli impregnated agar pellets and cecal ligation to model sepsis was changed to model with cecal ligation only because of high mortality rate in previous model.
- Voltage of 1800 was required to increase the transfection efficiency of the luciferase reporter gene into the rat model; a single application of plasmid was just as effective as a double dose of plasmid
- EP increased luciferase vector transfection efficiency 53-fold as compared to non-electroporated controls
- Luciferase vector expression was predominately localized in the epithelium when injected intradermally with EP administration

- EP alone showed no detrimental effect on healing parameters, such as wound areas and wound breaking strength
- EP-assisted KGF-1 plasmid DNA improved wound closure and quality of healing as compared to KGF-1 alone

Reportable Outcomes

List manuscripts, abstracts, presentations, patents, licenses etc

Publications and manuscripts

1. Brynes CK, Malone RW, Akhter N, Hilal S, Nass PH, Wetterwald A, Cecchini MG, Duncan MD, Harmon JW. Electroporation enhances transfection efficiency in murine cutaneous wounds. Wound Rep Reg 2004; 12: 397-403.
2. Lin MP, Marti GP, Dieb R, Wang J, Qaiser R, Bonde P, Duncan MD, Harmon JW. Electroporation Improves transfection efficiency in rat wound healing model. J Am College of Surgeons 199:S58, 2004. (Podium Presentation at the Surgical Forum, American College of Surgeons, 2004).
3. Marti GP, Lin, MP, Qaiser R, Dieb R, Wang J, Parth S, Bonde P, Duncan MD, Harmon JW. KGF-1 plasmid delivered with electroporation accelerates wound closure in diabetic mice. J Am College of Surgeons 199:S59, 2004. (Podium Presentation at the Surgical Forum, American College of Surgeons, 2004)
4. Marti G, Ferguson M, Wang J, Dieb R, Qaiser R, Bonde P Duncan MD, Harmon JW. Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model. Gene Therapy (A Nature Journal) 2004; 11: 1780-1785
5. Ferguson M, Byrnes CK, Sun E, Marti GP, Bonde P, Duncan MD, Harmon JW. Wound healing enhancement: Electroporation to address a classic problem of military medicine. World Journal of Surgery 2005; 29:S55-S59
6. Lin MP, Marti GP, Dieb R, Wang J, Ferguson M, Qaiser R, Bonde P, Duncan MD, Harmon JW. Delivery of Plasmid DNA Expression Vector for KGF-1 Using Electroporation to Improve Cutaneous Wound Healing in a Septic Rat Model. (Submitted to Wound Rep Reg)

Patents and licenses etc

1. Johns Hopkins University submitted a patent for the wound healing application of the electroporation technology. This patent was submitted prior to the award of the Army grant.
2. We established Canton Biotechnologies Inc in the state of Maryland to perform translational research.
3. Canton is negotiating a license of our electroporation-gene therapy patent from Johns Hopkins University.

Conclusions

Include importance, implications etc...Future work

Using DNA plasmid therapy to improve wound healing has been limited by low transfection efficiency. We used electroporation in a septic rat model of impaired wound healing to increase transfection efficiency. Electroporation increased transfection

efficiency up to 53-fold. Both the rate and quality of healing were improved with DNA plasmid expression vector for growth factor delivered with electroporation. These results demonstrated the capacity of electroporation-facilitated transfection with DNA plasmid expression vector for KGF-1 growth factor to improve wound healing in a sepsis model.

There are multiple implications for the use of electroporation in localized delivery of DNA plasmid expression vectors to wounded tissue, as the expression of any number of gene sequences can potentially be enhanced, including growth factors, hormones, developmental signaling peptides, and immunizing factors. An optimal approach may include a number of different expression products working together synergistically.

References

1. Robson, MC, Phillips, LG, Lawrence, WT et al. (1992) "The safety and effect of topically applied recombinant basic flbroblast growth factor on the healing of chronic pressure sores" Ann. Surg 216: 401-406
2. Robson, MC, Phillips, LG, Thomason, A et al. (1992) "Recombinant human platelet-derived growth factor-BB for the treatment of chronic pressure ulcers" Ann. Plast. Surg. 29: 193-201
3. Steed, DL (1995) "Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers" Diabetic Ulcer Study Group. J. Vasc. Surg 21: 71-78

Appendices

1. Brynes CK, Malone RW, Akhter N, Hilal S, Nass PH, Wetterwald A, Cecchini MG, Duncan MD, Harmon JW. Electroporation enhances transfection efficiency in murine cutaneous wounds. Wound Rep Reg 2004; 12: 397-403.
2. Ferguson M, Byrnes CK, Sun E, Marti GP, Bonde P, Duncan MD, Harmon JW. Wound healing enhancement: Electroporation to address a classic problem of military medicine. World Journal of Surgery 2005; 29:S55-S59
3. Lin MP, Marti GP, Dieb R, Wang J, Ferguson M, Qaiser R, Bonde P, Duncan MD, Harmon JW. Delivery of Plasmid DNA Expression Vector for KGF-1 Using Electroporation to Improve Cutaneous Wound Healing in a Septic Rat Model. (Submitted to Wound Rep Reg)

APPENDIX 1

Young Investigator Award Article

Electroporation enhances transfection efficiency in murine cutaneous wounds

COLMAN K. BYRNES, MB, BCh[b]; ROBERT W. MALONE, MD[c]; NABEEL AKHTER, MD[a]; PETRA H. NASS, PhD[a]; ANTOINETTE WETTERWALD[d]; MARCO G. CECCHINI[d]; MARK D. DUNCAN, MD[a]; JOHN W. HARMON, MD[a]

Transfection of wounds with DNA-encoding growth factors has the potential to improve healing, but current means of nonviral gene delivery are inefficient. Repeated high doses of DNA, necessary to achieve reliable gene expression, are detrimental to healing. We assessed the ability of in vivo electroporation to enhance gene expression. Full-thickness cutaneous excisional wounds were created on the dorsum of female mice. A luciferase-encoding plasmid driven by a CMV promoter was injected at the wound border. Following plasmid administration, electroporative pulses were applied to injection sites. Pulse parameters were varied over a range of voltage, duration, and number. Animals were euthanized at intervals after transfection and the luciferase activity measured. Application of electric pulses consistently increased luciferase expression. The electroporative effect was most marked at a plasmid dose of 50 μg, where an approximate tenfold increase was seen. Six 100-μs-duration pulses of 1750 V/cm were found to be the most effective in increasing luciferase activity. High numbers of pulses tended to be less effective than smaller numbers. This optimal electroporation regimen had no detrimental effect on wound healing. We conclude that electroporation increases the efficiency of transgene expression and may have a role in gene therapy to enhance wound healing. **(WOUND REP REG 2004;12:397–403)**

Exogenous application of growth factors has been shown to have the potential to improve wound healing.[1–3] However, the requirement for frequent dosing because of the short half-life of delivered peptides has limited their clinical efficacy. Gene therapy with growth factor-encoding DNA may allow the continuous production of these factors within wounds to encourage healing. The skin is an attractive potential target for gene therapy due to its accessibility for application and monitoring and the possibility of removing tissue if necessary.[4]

PEI	Polyethylenimine

Although virally mediated gene delivery has a high transfection efficiency, there are serious concerns about its safety and potential pathogenicity.[5] However, alternative means of nonviral in vivo gene delivery remain inefficient, limiting their value in therapeutic applications. The large DNA load and repeated applications necessary to achieve a therapeutic effect with naked DNA approaches have been shown to be detrimental to healing.[6] An effective means of increasing transfection efficiency may allow for a reduction in the amount of DNA required to produce a clinical effect and significantly improve the therapeutic impact.

Electroporation has been commonly used for the delivery of DNA to cells in vitro since the early 1980s.[7] Electroporation is the application of an electrical field across cells in order to permeabilize the cell membranes and allow the entry of macromolecules.[8] The applied electrical field increases transmembrane

From the Section of Surgical Sciences[a], Johns Hopkins Bayview Medical Center, Johns Hopkins Medical Institutions, Baltimore, Maryland; Queen's University[b], Belfast, Northern Ireland; MIST Institute/Gene Delivery Alliance[c], Rockville, Maryland; and Department of Clinical Research[d], University of Bern, Inselspital, Bern, Switzerland.

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Reprint requests: John W. Harmon, MD, Section of Surgical Sciences, Room 5C, "A" Building, Johns Hopkins Bayview Medical Center, 4940 Eastern Avenue, Baltimore, MD 21224. Fax: (410) 550-1274; Email: jharmon@jhmi.edu.

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voltage potential, exceeding membrane dielectric strength and causing membrane defects through which the charged polynucleotide may pass.[9] The electrophoretic effect of the field may also enhance DNA migration within tissues.[10] In vivo electroporation has been used to increase intracellular delivery of agents such as chemotherapeutics, both directly into tumors and also to enhance transdermal drug delivery.[9] Although most commonly used for in vitro transfection applications, electroporation has been of benefit in in vivo settings as well. Improvements in the transfection of liver,[11,12] muscle,[13,14] tumor,[15,16] and cutaneous tissue[17–20] have all recently been shown using electroporation. The prior skin experiments were carried out on normal, unwounded skin with the clinical goal of immunization.[21] The efficacy of this technique in abnormal, injured skin for use in potential wound healing experiments has not been reported. With this goal in mind, we carried out an extensive evaluation of electroporation-facilitated cutaneous transfection comparing different electrical parameters and dosage regimens. We also assessed duration of transfection and the effect of the electroporation protocol on wound healing.

MATERIALS AND METHODS

Female 6- to 8-week-old BALB-c mice were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Plasmid preparations

The plasmid gWIZ-Lux, containing a CMV promoter and luciferase transgene, was obtained from Gene Therapy Systems (San Diego, CA). Plasmids were purified using an endotoxin-free plasmid purification kit (Qiagen, Santa Clarita, CA) following culture in transformed DH-5α bacteria. Plasmids were stored at −70 °C at a concentration of 2 mg/ml until used.

Plasmid administration

Animals were anesthetized with an intraperitoneal injection of 0.02 ml/g of a 1.25 percent Avertin solution. Their dorsum was shaved and two symmetrical full-thickness excisional wounds created on their back using a 5-mm punch biopsy instrument. Fifty microliters of the appropriate concentration of luciferase plasmid was injected intradermally both anterior and posterior to each wound. The resulting skin blebs confirmed intradermal delivery of the plasmid and were marked with indelible ink. Wounds were left undressed and animals were housed individually. In addition to injection of naked plasmid, plasmid was also injected with the addition of Lipofectamine or DMRIE-C (Gibco-BRL, Carlsbad, CA), or polyethylenimine (PEI; Sigma-Aldrich, St. Louis, MO).

Electroporation

Animals were electroporated at the site of injection within 2 minutes of plasmid administration, using a square wave electroporator (ECM 830, BTX Genetronics, San Diego, CA). A custom designed pin electrode, consisting of two 10-mm rows of parallel needles separated by 5 mm was used to apply the electroporation voltage (Figure 1). Between 6 and 18 square wave pulses were administered, at an amplitude of between 400 and 1800 V, a duration of between 100 μs to 20 ms, and an interval between pulses of 125 ms (Figure 2).

In vitro luciferase assay

After at least 24 hours, animals were euthanized, and 25-mm^2 specimens at the marked injection sites were excised. The skin tissue was homogenized in a cell lysis buffer (Pharmingen, San Diego, CA) containing a proteinase inhibitor cocktail (Sigma-Aldrich), using a polytron homogenizer. Samples were centrifuged at 14,000 r.p.m. for 30 seconds before use. The luciferase activity of each sample was determined using a commercial luciferase assay kit (Pharmingen). Forty microliters of each sample was placed into a luminometer (Monolight 3010, BD Biosciences, San Jose, CA) with 100 μl of cofactor solution. Luciferase substrate (100 μl) was added and the photon emission measured over the following 10 seconds.

The protein concentration of each sample was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Light output was normalized to each sample's protein concentration and luciferase activity expressed as RLU/μg protein.

In vivo luciferase imaging

To assess the time course of luciferase expression with and without electroporation, animals were analyzed using an in vivo luciferase imaging system. In these experiments, mice were wounded and injected with plasmid as previously described, but only the injection sites on the right side of each animal were electroporated using six 100 μs pulses of 1750 V/cm, with an interval of 125 ms.

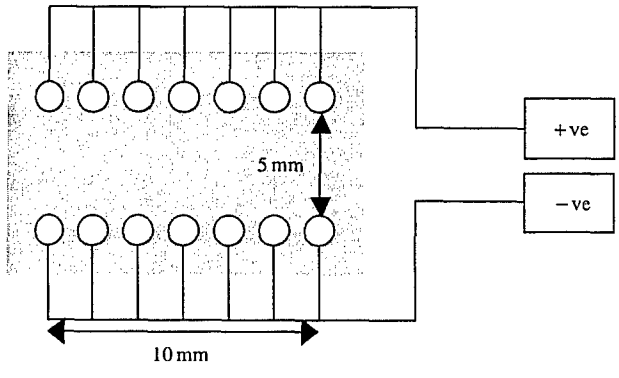

FIGURE 1. Schematic design of pin electrode.

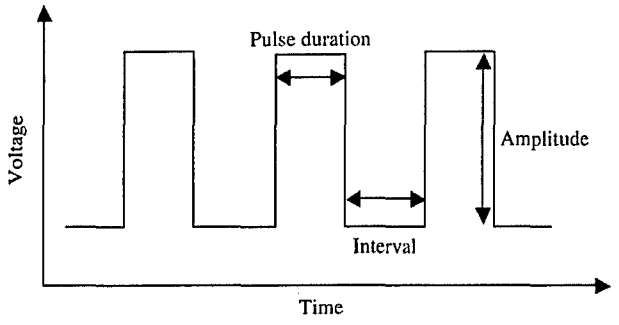

FIGURE 2. Square wave electroporation characteristics.

At time points after the initial transfection, animals were sedated with intraperitoneal Avertin, and then injected intraperitoneally with 150 mg/kg of D-luciferin in water. After a conventional light photograph was taken, bioluminescent images were acquired using a cooled charged coupled device camera (IVIS, Xenogen, Alameda, CA). Luminescent images were taken at intervals of between 10 and 40 minutes following luciferin administration, during which time the light emission had been shown to be in a plateau phase. Bioluminescent images were overlaid onto the conventional image of each animal, and the light emission, corrected for background luminescence, was calculated for each injection site using image analysis software (Living Image, Xenogen). The different spectral colors represent a linear scale of the intensity of luminescence, corresponding to the total number of photons emitted per second from a square cm of tissue (Figure 3). Activities are expressed as total photons per second for equal-sized regions of interest at the injection sites are shown graphically (Figure 4).

Wound healing measurements

Animals were anesthetized and wounded as before. No plasmid was administered, and half the wounds were electroporated with six 100-μs, 1750 V/cm square wave pulses of 100 μs duration. Animals were euthanized on day 7 following wounding. The wound eschar was carefully removed and the nonepithelialized wound border traced in situ onto clear acetate paper. Images were digitized at 600 dpi (Visioneer Paperport 6000, Visioneer, Fremont, CA) and wound areas were calculated using image analysis software based on NIH image (Scion Image, Frederick, MD). Areas were expressed as a pixel count. The dorsal skin was subsequently removed in the plane deep to the panniculus carnosus muscle. Skin strips were cut according to a 2×0.5 cm template with the wound at the midpoint. Each strip was loaded onto a custom built tensiometer and traction applied at a rate of 10 mm/minute until complete disruption of the wound occurred. The wound burst strength was recorded in Newtons as the peak force across the tissue prior to fracture.

Statistical analysis

Results were presented as means ± SEM. Differences in means between groups were analyzed for significance using Student's *t*-test or ANOVA as appropriate.

RESULTS

There was no evidence of luciferase activity in uninjected skin tissue sites. Enzymatic activity was detected at plasmid dosages as low as 0.1 μg plasmid. Increasing the dosage of plasmid injected caused the amount of luciferase activity to rise across the 500-fold range tested up to 50 μg (Figure 5).

Lipofection and polyfection

The addition of Lipofectamine (80 μl/ml), DMRIE (120 μl/ml), or PEI (5:1 ratio of PEI-Nitrogen: DNA-Phosphate) to plasmid solutions consistently reduced or abolished the luciferase activity seen in the skin tissue with 10 μg naked plasmid injection

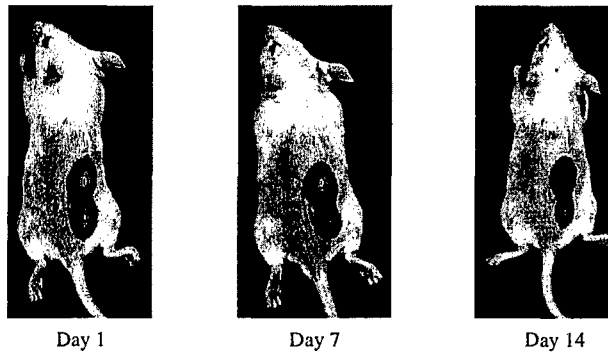

FIGURE 3. Serially acquired bioluminescent images of a single mouse after 50-μg injections of luciferase plasmid. The color violet represents a low level of luminescence through to red, indicating the highest intensity of luminescence. Only the wounds on the right side of the animal were electroporated. Images were taken on days 1, 7, and 14.

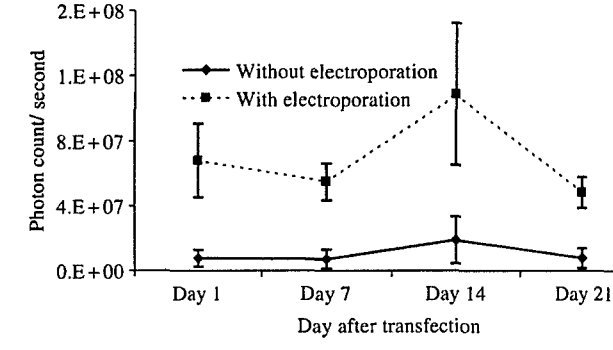

FIGURE 4. The time course of luciferase activity after a single naked plasmid injection, with and without electroporation compared to the unelectroporated group (n = 4 animals per group).

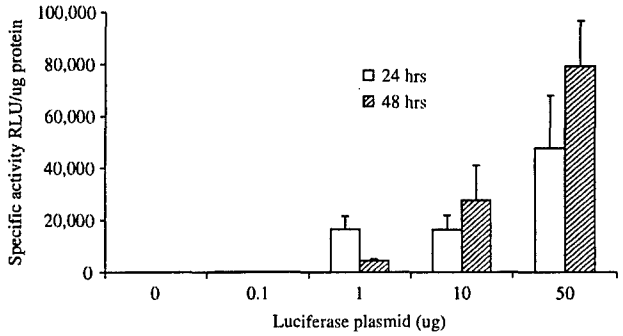

FIGURE 5. Luciferase plasmid dose-response curve in unelectroporated skin tissue.

(Figure 6). The highest luciferase activity was always evident in animals injected with naked plasmid, without either lipofection or polyfection.

Electroporation parameters

The application of electric pulses locally to the injection site consistently increased the transfection efficiency when measured at 24 hours postinjection. Increasing the applied voltage across the injected tissue caused an increase in the luciferase activity (Figure 7A). This effect was most apparent at higher plasmid doses where the increase was over tenfold. Higher voltages than 1800 V/cm tended to either cause arcing of the electric pulse between the electrodes or left some signs of an electrical burn on the animal's skin.

Increasing the number of electroporative pulses from 6 to 18 attenuated the increase in transfection efficiency (Figure 7B).

A low-voltage, long-duration series of pulses (6×20 ms, 400 V/cm) was not particularly effective in increasing transfection efficiency with 10 μg plasmid, when compared to high-voltage, short-duration pulses (6×100 μs, 1750 V/cm) Figure 7C. This is in contrast to skeletal muscle tissue with 10 μg plasmid, where low-voltage electroporation parameters caused a 20-fold increase in transfection efficiency (Figure 7D).

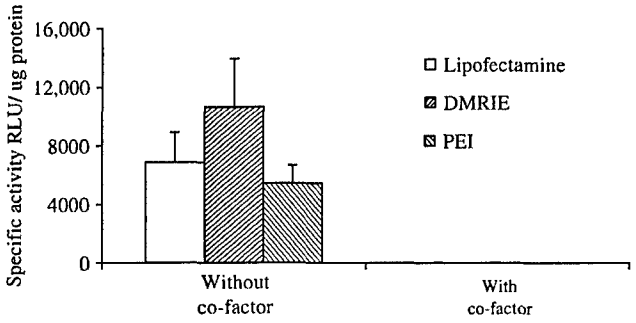

FIGURE 6. Plasmid injections with different adjuvants. Naked plasmid injection (10 μg) was found to be superior to either lipofection or polyfection when gene expression was measured 24 hours after plasmid injection.

Plasmid dose-response effect with optimal electroporation parameters

Using the optimal electroporation parameters, the electroporative effect was seen over a range of plasmid doses tested, but was most effective at higher doses of DNA. Using 50 μg of DNA, electroporation produced a large increase in luciferase activity. With electroporation, 10 μg of plasmid produced luciferase expression equivalent to that achieved with 50 μg of naked plasmid without electroporation (Figure 8).

Duration of transfection

Electroporation of the skin tissue consistently led to an increase in the transfection efficiency after a single injection of plasmid (Figure 3). In order to examine if electroporation had any effect on the duration of gene expression, animals were imaged at varying intervals after a single plasmid injection, between 1 day and 3 weeks (Figure 4). The luciferase activity was approximately tenfold higher ($7.71\times10^6 \pm 5.24\times10^6$ vs. $6.82\times10^7 \pm 2.28\times10^7$, $p < 0.01$ at day 1) in the electroporated injection sites than in the nonelectroporated sites. This increased activity was maintained throughout the duration of the experiment, up to an interval of 3 weeks.

Effect of electroporation on wound healing

Measurement of both the wound areas and wound breaking strength at day 7 in animals with and without the administration of the most effective electroporation settings (6×100 μs, 1750 V/cm), had no detrimental effect on these healing parameters. In fact, there was a slight nonsignificant tendency for the electroporated wounds to have improved healing as evidenced by a smaller open area and greater tensile strength (Figure 9A and B).

DISCUSSION

These experiments show that electroporation can improve plasmid transfection efficiency in cutaneous wound tissue. This effect was maximal, over tenfold, at the higher doses of plasmid administered to the wounds and at greater electroporation voltages. Using a series of high-voltage, short-duration pulses was found to be superior in efficacy to lower-voltage, longer-duration pulses. The electroporation protocol was not detrimental to wound healing.

The effect seen may be of considerable benefit in wound healing applications. Gene therapy has potential to treat a wide spectrum of both genetic and acquired diseases. The skin may be transfected in gene therapy

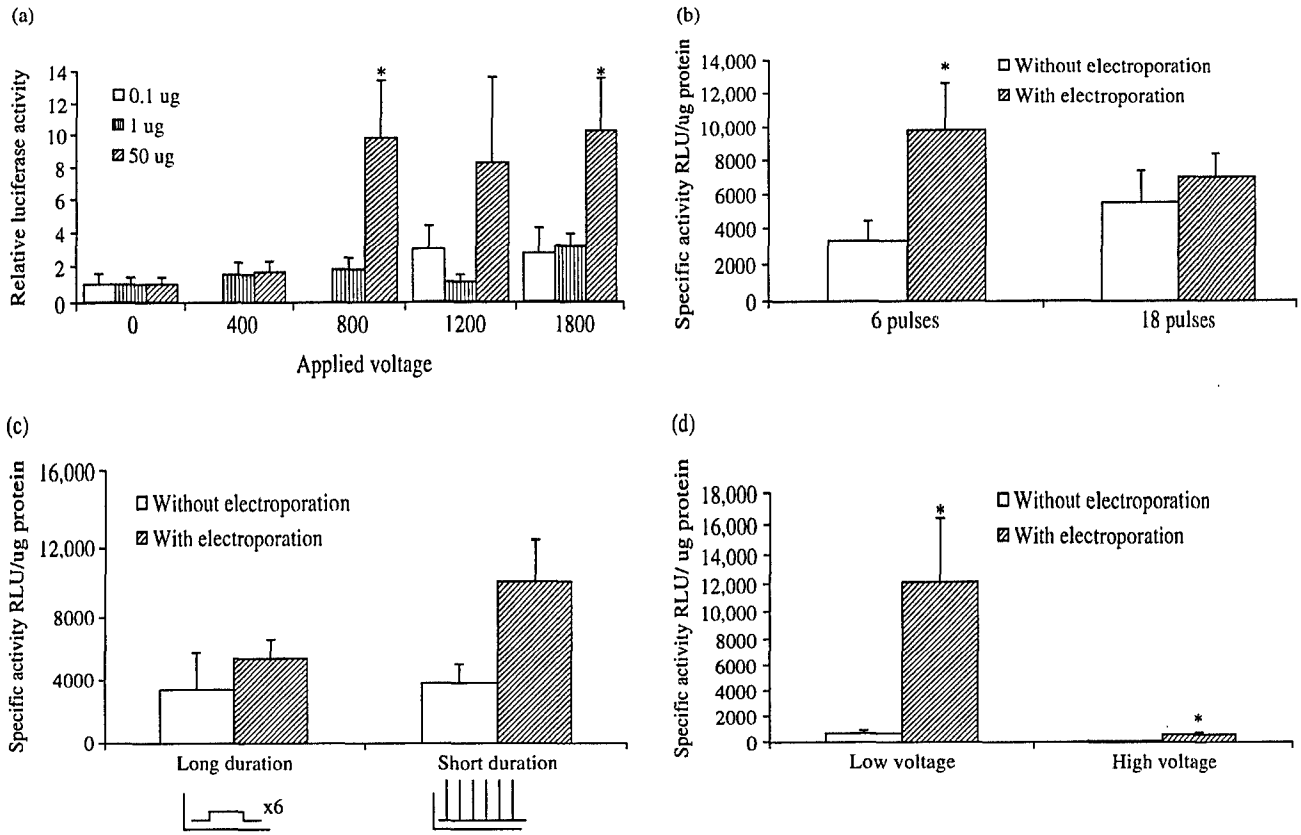

FIGURE 7. Optimization of electroporation parameters on luciferase activity. (A) The effect of increasing the electroporative amplitude, 24 hours after transfection, on the relative luciferase activity, at several concentrations of plasmid solution. Six pulses of the indicated voltage were applied, each with a duration of 100 μs and an interval of 125 ms ($n = 4$ animals per group, * $p < 0.05$ compared to the unelectroporated group. Kruskal–Wallis One Way ANOVA). (B) Six pulses at 1750 v/cm were more effective than 18 pulses with the same characteristics 24 hours after transfection ($n = 4$ animals per group, 10 μg plasmid * $p < 0.05$ compared to the unelectroporated group, Student's *t*-test). (C) A high-voltage, short-duration (1750 V/cm, 100 μs) series of six pulses tended to be more efficacious than low-voltage, longer-duration (400 V/cm, 20 ms) pulses ($n = 4$ animals per group, 10 μg plasmid). (D) Six low-voltage (200 V/cm), long-duration (20 ms) caused a 20-fold increase in luciferase activity in skeletal muscle tissue 24 hours after transfection ($n = 4$ animals per group, 10 μg plasmid. * $p < 0.01$ compared to the unelectroporated group, Student's *t*-test).

applications for both systemic treatment, such as immunization, as well as local therapy, including the enhancement of wound healing.[22] Ex vivo gene therapy techniques have been used in the field of wound healing,[23,24] but in vivo techniques have the advantage of being simpler and less time consuming, making them more appropriate for potential clinical use.[25]

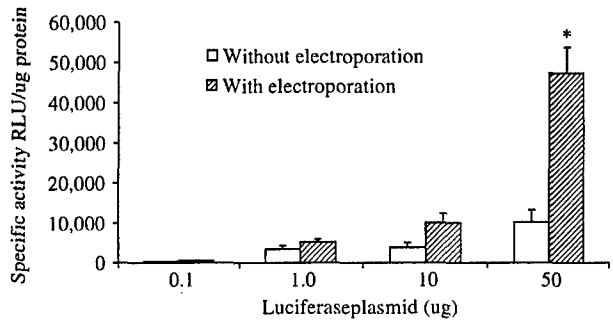

FIGURE 8. Effect of increasing the plasmid load on the efficacy of electroporation. Six 100-μs, 1750 V/cm pulses were given with an interval of 125 ms and luciferase activity measured at 24 hours ($n = 4$ animals per group, * $p < 0.05$ compared to the unelectroporated group, Student's *t*-test).

Prior experience in our lab and others has shown that the use of DNA plasmids encoding different growth factors can improve wound healing in animal models.[26–28] Currently, the main barrier for in vivo gene therapy is delivery of DNA molecules to tissues in such a manner that they are efficiently expressed.[29] Clearly, DNA must reach the nucleus to be expressed; however, exogenous DNA tends to be sequestered in the extracellular tissue or in the cell cytoplasm.[30,31] Viral gene delivery has the advantage of achieving nuclear entry with high transfection efficiencies, particularly in nondividing cells and in vivo. However there are serious concerns regarding the safety and immunogenicity of current viral mediators.

Numerous techniques have been described for nonviral transfection of skin and other tissues, including naked plasmid injection,[32,33] topical application,[34] biolistic delivery with a gene gun,[35] and microseeding.[36] However, in vivo transfection efficiency with these techniques remains several orders of magnitude less efficient than that of in vitro transfection.

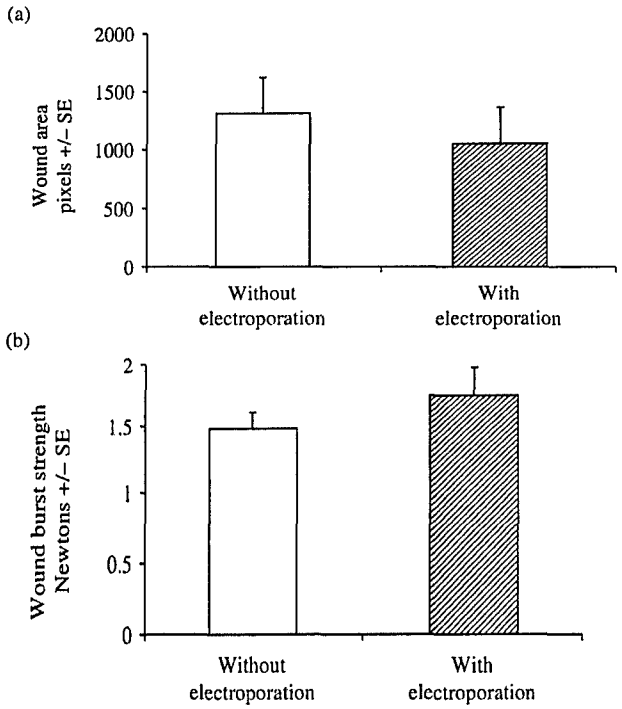

FIGURE 9. Effect of electroporation on wound healing parameters on day 7. (A) Wound areas in electroporated and unelectroporated wounds (n = 5 animals per group). (B) Wound breaking strengths (n = 5 animals per group).

Increasing gene expression with lipofection is effective in serum-free tissue culture settings, but not in the tissue setting. The liposomal agents bind to extracellular protein and actually prevent DNA uptake into cells. Interestingly, lipofection has been shown to be of some benefit following intraluminal delivery of plasmid into hollow visci, including blood vessels,[37,38] the lung,[39] and colon.[40] However, we found that in skin, the liposomal agents used had a detrimental effect on transfection efficiency when compared to the injection of naked plasmid alone. Prior reports have also suggested that lipofection or polyfection may not be advantageous in skin tissue.[41,42]

It is interesting to compare the effects of electroporation in skin with its effects in other tissues. Muscle seems to be the ideal target for in vivo electroporation. It is suggested that the large size of striated muscle cells gives them properties that interact favorable with an electrical field. Increases in transfection efficiency of 2–4 log with relatively low-voltage electrical fields have been achieved in striated muscle.[13] Our results in skin are modest in comparison.

In this study, we demonstrated that electroporation is a simple, safe, and efficacious means of improving transfection efficiency in skin wounds. The application of high-voltage, short-duration, square wave electrical field pulses to wounded tissue can enhance gene expression over tenfold. With this approach the dose of plasmid can therefore potentially be reduced tenfold as compared to what has been required with naked plasmid. This decrease in the dose of DNA application is important as it is likely to diminish the detrimental effect on wound healing seen with high doses of DNA that we have reported previously.[6] In combination with one or more appropriate transgene(s)-encoding growth factors, electroporation has considerable potential in cutaneous wound healing applications.

ACKNOWLEDGMENTS

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REFERENCES

1. Robson MC, Phillips LG, Lawrence WT, Bishop JB, Youngerman JS, Hayward PG, Broemeling LD, Heggers JP. The safety and effect of topically applied recombinant basic fibroblast growth factor on the healing of chronic pressure sores. Ann Surg 1992;216: 401–6.
2. Steed DL. Diabetic Ulcer Study Group. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers. J Vasc Surg 1995; 21:71–8.
3. Robson MC, Phillips LG, Thomason A, Altrock BW, Pence PC, Heggers JP, Johnston AF, McHugh TP, Anthony MS, Robson LE et al. Recombinant human platelet-derived growth factor-BB for the treatment of chronic pressure. Ann Plast Surg 1992;29: 193–201.
4. Greenhalgh DA, Rothnagel JA, Roop DR. Epidermis: an attractive target tissue for gene therapy. J Invest Dermatol 1994;103 (5 Suppl.):63S–9S.
5. Felgner PL, Rhodes G. Gene therapeutics. Nature 1991;349:351–2.
6. Byrnes CK, Khan FH, Nass PH, Hatoum C, Duncan MD, Harmon JW. Success and limitations of a naked plasmid transfection protocol for keratinocyte growth factor-1 to enhance cutaneous wound healing. Wound Rep Reg 2001;9:341–6.
7. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1982;1:841–5.
8. Somiari S, Glasspool-Malone J, Drabick JJ, Gilbert RA, Heller R, Jaroszeski MJ, Malone RW. Theory and in vivo application of electroporative gene delivery. Mol Ther 2000;2:178–87.
9. Prausnitz MR, Bose VG, Langer R, Weaver JC. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. Proc Natl Acad Sci USA 1993;90:10504–8.
10. Satkauskas S, Bureau MF, Puc M, Mahfoudi A, Scherman D, Miklavcic D, Mir LM. Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. Mol Ther 2002;5:133–40.
11. Heller R, Jaroszeski M, Atkin A, Moradpour D, Gilbert R, Wands J, Nicolau C. In vivo gene electroinjection and expression in rat liver. FEBS Lett 1996;389:225–8.
12. Suzuki T, Shin BC, Fujikura K, Matsuzaki T, Takata K. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett 1998;425:436–40.
13. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci USA 1999;96:4262–7.

14. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther 1999;6:508–14.
15. Heller L, Jaroszeski MJ, Coppola D, Pottinger C, Gilbert R, Heller R. Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo. Gene Ther 2000;7:826–9.
16. Wells JM, Li LH, Sen A, Jahreis GP, Hui SW. Electroporation-enhanced gene delivery in mammary tumors. Gene Ther 2000;7:541–7.
17. Titomirov AV, Sukharev S, Kistanova E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. Biochim Biophys Acta 1991;1088:131–4.
18. Glasspool-Malone J, Somiari S, Drabick JJ, Malone RW. Efficient nonviral cutaneous transfection. Mol Ther 2000;2:140–6.
19. Drabick JJ, Glasspool-Malone J, King A, Malone RW. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 2001;3:249–55.
20. Heller R, Schultz J, Lucas ML, Jaroszeski MJ, Heller LC, Gilbert RA, Moelling K, Nicolau C. Intradermal delivery of interleukin-12 plasmid DNA by in vivo electroporation. DNA Cell Biol 2001;20: 21–6.
21. Dujardin N, Van Der Smissen P, Preat V. Topical gene transfer into rat skin using electroporation. Pharm Res 2001;18:61–6.
22. Eming SA, Morgan JR, Berger A. Gene therapy for tissue repair: approaches and prospects. Br J Plast Surg 1997;50:491–500.
23. Vogt PM, Thompson S, Andree C, Liu P, Breuing K, Hatzis D, Brown H, Mulligan RC, Eriksson E. Genetically modified keratinocytes transplanted to wounds reconstitute the epidermis. Proc Natl Acad Sci USA 1994;91:9307–11.
24. Rosenthal FM, Cao L, Tanczos E, Kopp J, Andree C, Stark GB, Mertelsmann R, Kulmburg P. Paracrine stimulation of keratinocytes in vitro and continuous delivery of epidermal growth factor to wounds in vivo by genetically modified fibroblasts transfected with a novel chimeric construct. In Vivo 1997;11:201–8.
25. Vogel JC. Nonviral skin gene therapy. Hum Gene Ther 2000;11:2253–9.
26. Andree C, Swain WF, Page CP, Macklin MD, Slama J, Hatzis D, Eriksson E. In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. Proc Natl Acad Sci USA 1994;91:12188–92.
27. Sun L, Xu L, Chang H, Henry FA, Miller RM, Harmon JM, Nielsen TB. Transfection with aFGF cDNA improves wound healing. J Invest Dermatol 1997;108:313–8.
28. Eming SA, Whitsitt JS, He L, Krieg T, Morgan JR, Davidson JM. Particle-mediated gene transfer of PDGF isoforms promotes wound repair. J Invest Dermatol 1999;112:297–302.
29. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 1995;270:18997–9007.
30. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum Gene Ther 2001;12: 861–70.
31. Byrnes CK, Nass PH, Shim J, Duncan MD, Lacy B, Harmon JW. Novel nuclear shuttle peptide to increase transfection efficiency in esophageal mucosal cells. J Gastrointest Surg 2002;6:37–42.
32. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. Science 1990;247 (4949 Part 1):1465–8.
33. Hengge UR, Walker PS, Vogel JC. Expression of naked DNA in human, pig, and mouse skin. J Clin Invest 1996;97:2911–6.
34. YuWH, Kashani-Sabet M, Liggitt D, Moore D, Heath TD, Debs RJ. Topical gene delivery to murine skin. J Invest Dermatol 1999;112:370–5.
35. Williams RS, Johnston SA, Riedy M, DeVit MJ, McElligott SG, Sanford JC. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA 1991;88:2726–30.
36. Eriksson E, Yao F, Svensjo T, Winkler T, Slama J, Macklin MD, Andree C, McGregor M, Hinshaw V, Swain WF. In vivo gene transfer to skin and wound by microseeding. J Surg Res 1998;78: 85–91.
37. Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. Science 1993;261:209–11.
38. Liu Y, Liggitt D, Zhong W, Tu G, Gaensler K, Debs R. Cationic liposome-mediated intravenous gene delivery. J Biol Chem 1995;270:24864–70.
39. Fortunati E, Bout A, Zanta MA, Valerio D, Scarpa M. In vitro and in vivo gene transfer to pulmonary cells mediated by cationic liposomes. Biochim Biophys Acta 1996;1306:55–62.
40. Liptay S, Weidenbach H, Adler G, Schmid RM. Colon epithelium can be transiently transfected with liposomes, calcium phosphate precipitation and DEAE dextran in vivo. Digestion 1998;59: 142–7.
41. Udvardi A, Kufferath I, Grutsch H, Zatloukal K, Volc-Platzer B. Uptake of exogenous DNA via the skin. J Mol Med 1999;77:744–50.
42. Meuli M, Liu Y, Liggitt D, Kashani-Sabet M, Knauer S, Meuli-Simmen C, Harrison MR, Adzick NS, Heath TD, Debs RJ. Efficient gene expression in skin wound sites following local plasmid injection. J Invest Dermatol 2001;116:131–5.

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APPENDIX 2

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Wound Healing Enhancement: Electroporation to Address a Classic Problem of Military Medicine

Mark Ferguson, Ph.D.,[1] Colman Byrnes, M.B. Ch.B.,[2] Leon Sun, M.D., Ph.D.,[3] Guy Marti, M.D.,[4] Pramod Bonde, M.D.,[1] Mark Duncan, M.D.,[1] John W. Harmon, M.D.[1]

[1]Section of Surgical Sciences, Room 5C, A' Building, Johns Hopkins Bayview Medical Center, Johns Hopkins Medical Institutions, 4940 Eastern Avenue, 21224, Baltimore, Maryland, USA
[2]Queen's University Belfast, Northern Ireland
[3]Uniformed Services University of the Health Sciences, 20889, Bethesda, Maryland, USA
[4]St. Jean Clinic and Hospital, Melun, France

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Abstract. The major goal of wound healing biology is to determine how a wound can be induced to repair damaged tissue faster and more efficiently. Enhancement of dermal and epidermal regeneration is an extremely important goal for the treatment of many different types of wounds. Exogenous application of growth factors to the wound site has been shown to have potential to improve wound healing. Frequent applications of large amounts of growth factor have been required. This is because proteases in the wound quickly destroy peptide growth factor. Gene therapy has the potential to produce growth factors deep within the wound, where they can be effective as well as able to constantly replenish growth factor that is destroyed by peptidases. We have shown that application of plasmid DNA expression vectors directly into the wound is an inefficient modality. Electroporation, the application of an electrical field across cells to permeabilize the cell membrane has led us to explore the possibility of utilizing the technique to enhance transfection efficiency. We have identified electroporation parameters that improve the efficiency of DNA transfection in cutaneous wounds, and we have shown that electroporation itself does not impair wound healing. We are now on the threshold of exploring whether electroporation-assisted transfection with DNA plasmid expression vectors for growth factors will be an effective modality for enhancing cutaneous wound healing.

The major goal of wound healing biology is to determine how a wound can be induced to repair the damaged tissues faster and more efficiently. Enhancement of dermal and epidermal regeneration is an extremely important goal for the treatment of many different types of wounds.

Wound healing occurs in multiple phases, with each phase controlled by growth factors. Although each growth factor is only present in a small amounts, they are believed to exert a powerful influence on wound repair. In 1986 the Nobel Prize in medicine was awarded jointly to Rita Levi-Montalcini and Stanley Cohen for their discoveries of growth factors. They characterized nerve growth factor (NGF) and epidermal growth factor (EGF), respectively [1]. The importance of growth factors during the wound healing process cannot be overstated. They act as triggers that stimulate sedentary cell lineages to proliferate and ultimately lay down new cells at the wound site. Over 18 different types of growth factors have been found at wound sites [2], including EGF, TGF-α, FGF 1-10, KGF (A.K.A. FGF7), PDGF, IGF-1, VEGF, TGFβ1-3, CTGF, Activin, DL-1α & β, TNF-α, TGF-β and HIF1-α.

Topical Application of Growth Factors

Exogenous application of growth factors to the wound site is an area of interest to many researchers and has been shown to have the potential to improve wound healing [3–5]. Recombinant PDGF (Regranex, Ortho-McNeil) has been licensed and approved by the U.S. Food and Drug Administration as a topically applied gel that acts biologically in much the same way as endogenous PDGF by promoting the chemotactic recruitment and proliferation of cells involved in wound repair. Regranex is indicated for the treatment of diabetic neuropathic ulcers [6]. Enhanced healing was observed with topically applied PDGF in the clinical trial. But the effect was minimal, with a marginal increase in the incidence of complete healing as compared to a placebo gel (50% vs 37%).

Topical application of growth factors to wounds has major drawbacks; for example, in our animal study of exogenously applied FGF-1 it was necessary to repeatedly administer large amounts of recombinant FGF-1 to achieve a significant improvement in wound healing [7]. Because of the short half-life of the reagent, large amounts of FGF-1 were required raising the costs and complexity of its potential therapeutic use; furthermore the wound eschar can block access to the wound by growth factors. Although many reports exist on the topical application of growth factors on wounds in humans there are only a few studies in which a positive effect was noted [8].

Norman Rich MD, FACS. Feistschrift. April 2004. USUHS.

Correspondence to: John W. Harmon, M.D., e-mail: jharmon@jhmi.edu

Table 1. Time course effects of aFGF and aFGF cDNA on wound areas.[a]

Day after wounding	aFGF (% control[b])	PBS Control (mm^2)	n[c]	aFGF cDNA (% control[b])	Control cDNA (mm^2)	n[d]
1	101 ± 4	36.4 ± 3.2	10	100 ± 2	30.5 ± 1.2	25
3	97 ± 6	36.5 ± 3.5	10	100 ± 3	32.6 ± 1.2	25
5	78 ± 13	37.2 ± 2.6	10	94 ± 3	32.3 ± 1.2	25
7	78 ± 12	28.6 ± 3.5	10	103 ± 6	24.3 ± 1.4	25
9	61 ± 12[e]	22.9 ± 3.4	10	106 ± 12	16.1 ± 1.1	25
11	62 ± 12[e]	16.5 ± 3.4	10	79 ± 11	10.7 ± 0.8	25
13	48 ± 11[e]	15.0 ± 3.0	10	56 ± 9[e]	8.8 ± 1.0	25
15	28 ± 8[e]	10.8 ± 2.9	10	42 ± 7[e]	5.3 ± 0.8	25

[a]Wound areas were measured in db/db mouse excision wounds treated with 1.0 μg of human recombinant aFGF (aFGF), PBS (control of aFGF group), 2.5 μg of pMEXneo-sp-aFGF (aFGF cDNA) or 2.5 μg pMEX-neo (control cDNA, control of aFGF cDNA group).
[b]Means ± SEM of the wound areas were expressed as a percentage of the areas of control wounds.
[c]Number of mice in each group (human recombinant aFGF or PBS control) [d]Number of mice in each group (pMEXneo-sp-aFGF or pMEXneo). Each group or experimental group consisted of five animals per experiment.
[e]$p < 0.05$, compared with the value with day 1 by the one-way analysis of variance test.

Gene Therapy Alternative

With the completion of the Human Genome Sequencing Project, the identification and cloning of potential growth factor genes yields the possibility of selectively expressing a gene at the wound site using a plasmid vector. Our group and others have previously reported enhancement of cutaneous wound healing following the transfection of tissue with plasmid vectors expressing the DNA for growth factors [7, 9, 10]. The major advantage of this technique is that gene therapy allows continuous production of the agent deep within the wound.

The central concern of gene therapy is determining how to deliver an intact and functional copy of the gene to the tissue of interest. Gene transfer techniques have included particle-mediated transfer of cDNA where the plasmid vector is coated onto gold beads and fired through the skin with a gene gun [11] or simply by infecting naked plasmid DNA directly to the tissue.

In our initial study [7], we used recombinant aFGF peptide applied topically or aFGF cDNA plasmid expression vector injected daily for 9 days into the edges of the wound. We sought to determine the effect on the wound healing rate of excisional wounds made on female diabetic mice. As shown in Table 1, accelerated wound closure was noted in both applications. This established the feasibility of enhancing wound healing by naked plasmid transfection in our hands.

Further studies, however, identified a major problem with the naked plasmid approach. We discovered that to achieve consistent transfection, high DNA plasmid load (100 μg per day) and repeated penetrations of the wound by separate injections were required to maintain measurable gene expression beyond two days [12]. When we compared KGF-containing vector with a control empty DNA vector, the KGF vector was more effective at accelerating wound healing [12]. However, wounded animals that received no injection demonstrated equivalent healing to animals that received KGR-bearing plasmid DNA (Fig. 1). This study indicated that simply injecting naked DNA into the wound was detrimental to wound healing and that both the repeated injections and high DNA load seemed to impair wound healing.

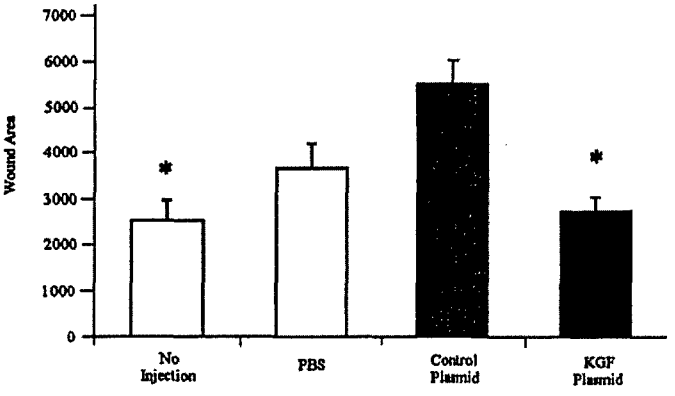

Fig. 1. Comparison of wounds treated with plasmids or various controls. Effect of treatment on wound areas was used as a measure of efficacy in the four experimental groups. $*p < 0.05$ compared to the control plasmid injection, ($n \geq 7$ per group).

Alternative Transfection Strategies—Electroporation

Increasing transfection efficiency may allow a reduction in the amount of DNA required to produce a clinical effect and significantly improve the therapeutic impact. Although virally mediated gene delivery has high transfection efficiency, there are serious concerns about its safety and potential pathogenicity [13]. The gene gun approach provides variable results and as outlined earlier the large DNA load and repeated applications necessary to achieve a therapeutic effect with naked DNA approaches have been shown to be disadvantageous to healing [12].

We decided to explore the use of electroporation to improve transfection efficiency. Electroporation has been commonly used for the delivery of DNA to cells in vitro since the early 1980s [14]. Electroporation is the application of an electrical field across cells in order to permeabilize the cell membranes and allow the entry of macromolecules [15]. The applied electrical field increases transmembrane voltage potential, exceeding membrane dielectric strength, and creating membrane pores through which the charged polynucleotide may pass [16]. The electrophoretic effect of the field may also enhance DNA migration within tissues [17]. In vivo electroporation has been used to increase intracellular delivery of agents such as chemotherapeutics directly into tumors, and also as a means of enhancing transdermal drug delivery [16]. Although most commonly used for in vitro transfection applications, electroporation has been of benefit in in vivo settings as well. Improvements in the transfection of liver [18, 19], muscle [20, 21], tumor [22, 23], and cutaneous tissue [24–27] have all recently been demonstrated using electroporation. Prior skin

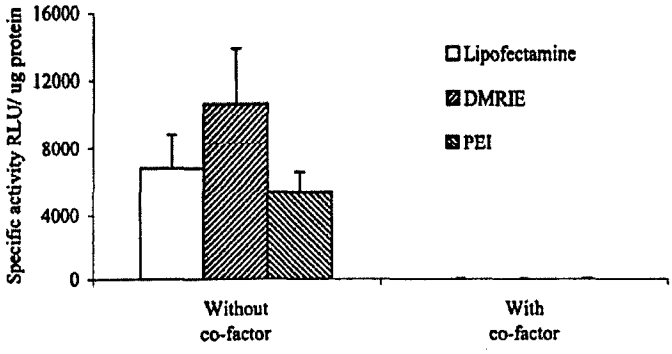

Fig. 2. Naked plasmid injection (10 μg) was found to be superior to either lipofection or polyfection when gene expression was measured 24 hours after plasmid injection.

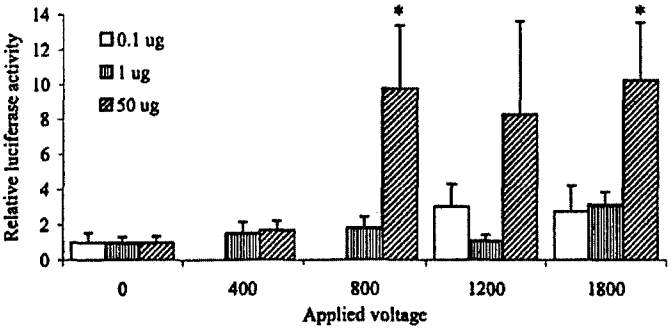

Fig. 3. The effect of increasing the electroporative amplitude, 24 hours after transfection, on the relative luciferase activity at several concentrations of plasmid solution. Six pulses of the indicated voltage were applied, each with a duration of 100 μs and an interval of 125 ms (n = 4 animals per group, *=$p < 0.05$ compared to the unelectroporated group. Kruskal-Wallisne one-way ANOVA).

experiments were carried out on normal unwounded skin with the clinical goal of immunization [28]. The efficacy of this technique in abnormal, injured skin for use in potential wound healing experiments had not been reported.

In a recent study from our group, we carried out extensive evaluation of electroporation-facilitated cutaneous transfection comparing different electrical parameters and dosage regimens. We also assessed the duration of transfection and the effect of the electroporation protocol on wound healing. Using a luciferase-based reporter vector, we determined the inefficiency of precomplexing the DNA with liposomes prior to transfection and electroporation (Fig. 2). We observed that electroporation after transfection with naked plasmid DNA offers significantly improved in vivo expression of a transfected plasmid (Fig. 3). The advantages of electroporation after transfection with naked plasmid DNA can clearly be seen in Figure 4, where with low-voltage electroporation the efficiency of luciferase expression was many times higher than without electroporation. It can also be clearly observed from a whole mouse luciferase image that expression of DNA encoding luciferase was much improved when electroporation was applied immediately after injection of DNA as compared to when electroporation was not applied (Fig. 5).

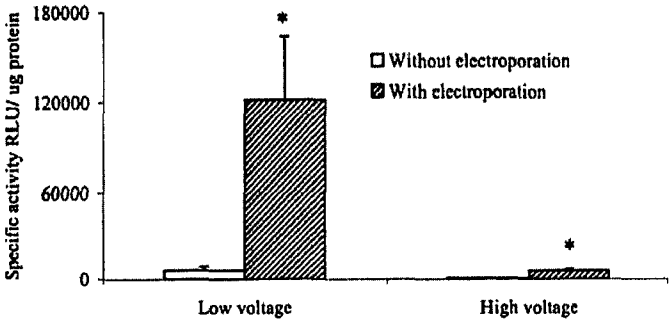

Fig. 4. Six low-voltage (200 V/cm), long-duration (20 ms) pulses caused a 20 fold increase in luciferase activity in skeletal muscle tissue 24 hours after transfection (n = 4 animals per group, 10 μg plasmid). *=$p < 0.01$ compared to the unelectroporated group, Student's t-test).

Conclusions

The potential therapeutic effects of growth factors to accelerate wound healing are clearly limited in the setting of topical application. At present the only available growth factor in wound healing therapy is topically applied PDGF (Regranex). Clinical trial results show that its effect is minimal. The benefits of selectively expressing a growth factor in vivo by efficiently delivering a plasmid DNA expression vector directly into the damaged tissue may improve and widen the therapeutic uses of growth factors as wound healing triggers. It is hoped that use of electroporation transfection technology with continuous production of growth factors at the wound site will greatly accelerate the wound healing process.

Acknowledgments

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References

1. Weltman JK. The 1986 Nobel Prize for Physiology or Medicine awarded for discovery of growth factors: Rita Levi-Montalcini, M.D., and Stanley Cohen, Ph.D. N. Engl. Reg. Allergy Proc 1987;8:47–48
2. Martin P. Wound healing—aiming for perfect skin regeneration. Science 1997;276:75–81
3. Robson MC, Phillips LG, Lawrence WT, et al. The safety and effect of topically applied recombinant basic fibroblast growth factor on the healing of chronic pressure sores. Ann. Surg 1992;216:401–406
4. Robson MC, Phillips LG, Thomason A, et al. Recombinant human platelet-derived growth factor-BB for the treatment of chronic pressure ulcers. Ann. Plast. Surg. 1992;29:193–201
5. Steed DL. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers. Diabetic Ulcer Study Group. J. Vasc. Surg 1995;21:71–78
6. Ortho-McNeil. Prescribing information for Regranex Gel. 1999;1–2
7. Sun L, Xu L, Chang H, et al. Transfection with aFGF cDNA improves wound healing. J. Invest. Dermatol 1997;108:313–318
8. Steed DL. Modifying the wound healing response with exogenous growth factors. Clin. Plast. Surg 1998;25:397–405
9. Andree C, Swain WF, Page CP, et al. In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. Proc. Natl. Acad. Sci. USA 1994;91:12188–12192
10. Eming SA, Morgan JR, Berger A. Gene therapy for tissue repair: approaches and prospects. Br. J. Plast. Surg 1997;50:491–500
11. Eming SA, Whitsitt JS, He L, et al. Particle-mediated gene transfer of PDGF isoforms promotes wound repair. J. Invest. Dermatol 1999;112:297–302

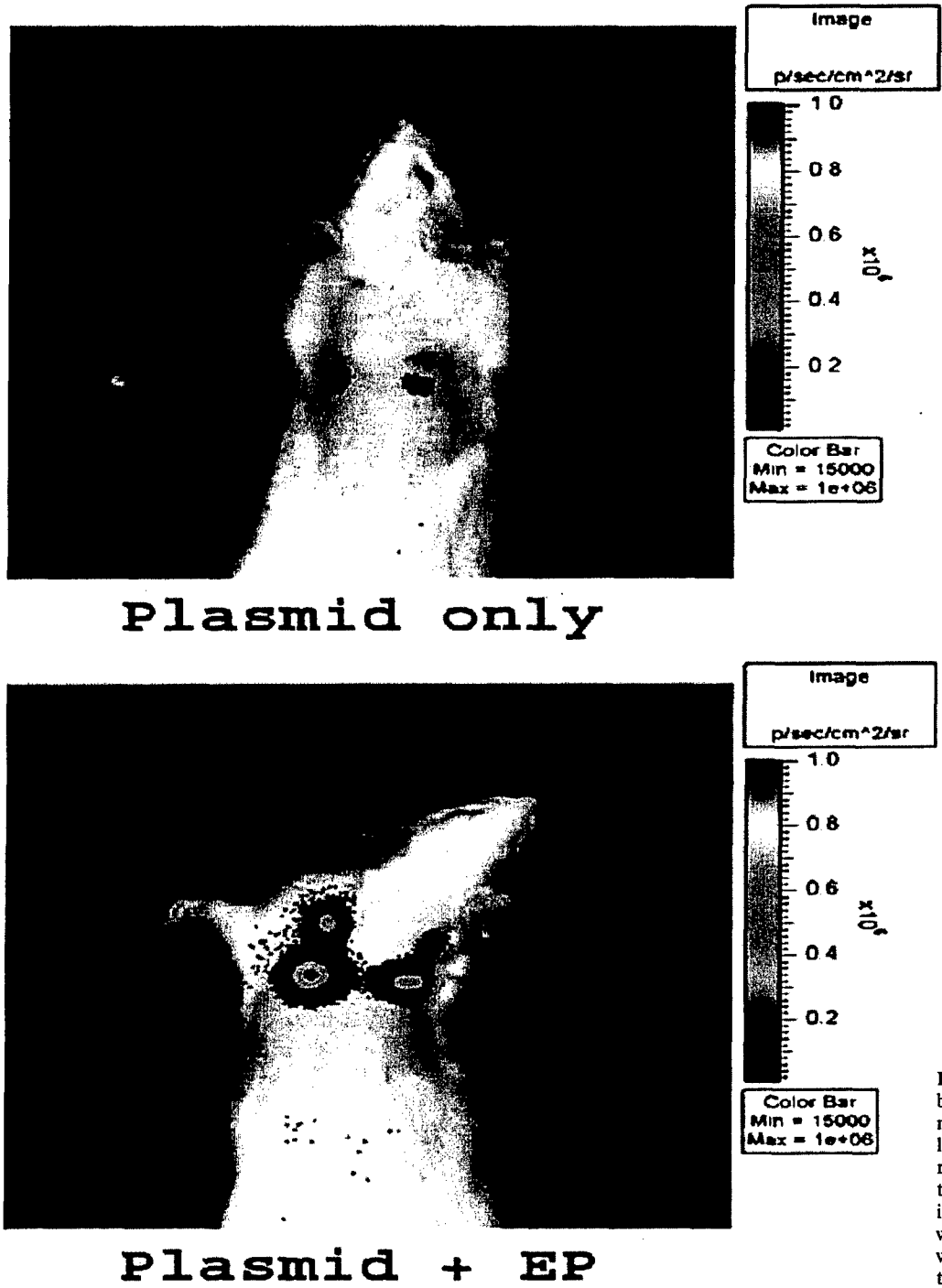

Fig. 5. Serially acquired bioluminescent images of a single mouse after 50 μg injections of luciferase plasmid. The color violet represents a low level of luminescence through to red, indicating the highest intensity of luminescence. Only the wounds on the right side of the animal were electroporated. Images were taken on days 1, 7, and 14.

12. Byrnes CK, Khan FH, Nass PH, et al. Success and limitations of a naked plasmid transfection protocol for keratinocyte growth factor-1 to enhance cutaneous wound healing. Wound Repair Regen 2001;9:341–346
13. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature 2003;4:346–358
14. Neumann E, Schaefer-Ridder M, Wang Y, et al. Gene transfer into mouse myloma cells by electroporation in high electric fields. EMBO J 1982;1:841–845
15. Somiari S, Glasspool-Malone J, Drabick JJ, et al. Theory and in vivo application of electroporative gene delivery. Mol. Ther 2000;2:178–187
16. Prausnitz MR, Bose VG, Langer R, et al. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. Proc. Natl. Acad. Sci. USA 1993;90:10504–10508
17. Satkauskas S, Bureau MF, Puc M, et al. Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. Mol. Ther 2002;5:133–140
18. Heller R, Jaroszeski M, Atkin A, et al. In vivo gene electroinjection and expression in rat liver. FEBS Lett 1996;389:225–228
19. Suzuki T, Shin BC, Fujikura K, et al. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett 1998;425:436–440
20. Mir LM, Bureau MF, Gehl J, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc. Natl. Acad. Sci. USA 1999;96:4262–4267
21. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther. 1999;6:508–514

22. Heller L, Jaroszeski MJ, Coppola D, et al. Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo. Gene Ther. 2000;1:826–829
23. Wells JM, Li LH, Sen A, et al. Electroporation-enhanced gene delivery in mammary tumors. Gene Ther. 2000;7:541–547
24. Titomirov AV, Sukharev S, Kistanova E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. Biochim. Biophys. Acta 1991;1088:131–134
25. Glasspool-Malone J, Somiari S, Drabick JJ, et al. Efficient nonviral cutaneous transfection. Mol Ther 2000;2:140–146
26. Drabick JJ, Glasspool-Malone J, King A, et al. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 2001;3:249–255
27. Heller R, Shultz J, Lucas ML, et al. Intradermal delivery by interleukin-12 plasmid DNA by in vivo electroporation. DNA Cell Biol 2001;20:21–26
28. Dujardin N, Van Der Smissen PVan Der , Preat V. Topical gene transfer into rat skin using electroporation. Pharm. Res 2001;18:61–66

Delivery of Plasmid DNA Expression Vector for KGF-1 Using Electroporation to Improve Cutaneous Wound Healing in a Septic Rat Model

Michael P Lin MS, Guy P Marti MD, Rami Dieb MD, Jiaai Wang BS, Mark Ferguson PhD, Rabia Qaiser MD, Pramod Bonde MD MS FRCS, Mark D Duncan MD FACS, John W Harmon MD FACS

Section of Surgical Sciences, Johns Hopkins Bayview Medical Center, Johns Hopkins Medical Institutions, Baltimore, MD, 21224

Correspondence and reprints: John W Harmon MD, Section of Surgical Sciences, Room 5C, 'A' Building, Johns Hopkins Bayview Medical Center, 4940 Eastern Avenue, Baltimore, MD 21224, USA. Phone: (410) 550-0401; Fax: (410) 550 1274; e-mail: jharmon@jhmi.edu

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Abstract

Objective

To further characterize the effects of DNA plasmid expression vectors delivered to rats using electroporation in a sepsis-based model of impaired wound healing.

Background

We have shown electroporation (EP) *in vivo* can enhance transfection efficiency and improve wound healing with DNA expression vectors for growth factors in a diabetic mouse model of impaired wound healing.[1]

Methods

To assess plasmid transfection and wound healing, gWIZ luciferase vector and PCDNA3.1/KGF-1 expression vectors were used respectively. Cutaneous wounds were produced using a 8mm-punch biopsy in Sprague Dawley rats. Healing was impaired by cecal ligation induced sepsis. We used NIH image analysis software and histologic assessment to assess wound closure.

Results

Plasmid Transfection: EP effectively increased expression of gWIZ luciferase vector up to 53-fold compared to vector without EP ($p<0.001$). Transfection was localized to cutaneous tissue when plasmid injection and EP were applied to the skin with skin specific parameters.

Wound Healing: The rate of wound healing was impaired by partial cecal ligation to induce sepsis. Average wound area was 4.7 times larger on day 9 in septic rats compared to control ($p<0.001$). The rate of wound healing improved with PCDNA3.1/KGF-1 expression vector and

EP. Wounds were 60% smaller on day 12 versus vector without EP ($p<0.009$). Quality of healing KGF-1 vector+EP scored 3.0 +/- 0.3 and was significantly better than that of 1.8 +/- 0.3 for treatment with vector alone ($p<0.05$).

Conclusions

Both the rate and quality of healing were improved with DNA plasmid expression vector for growth factor delivered with electroporation to septic rats.

Short Abstract:

Experiments were performed to characterize the effects of DNA plasmid expression vectors delivered to the rat using electroporation in a sepsis-based model of impaired wound healing. Both the rate and quality of healing were improved with DNA plasmid expression vector for KGF-1 growth factor delivered with electroporation.

Keywords

Wound healing, electroporation, keratinocyte growth factor, septic, gene therapy, rat

Introduction

Systemic sepsis has been shown to impair wound healing. [2-6] The exogenous delivery of growth factors *in vivo* has been demonstrated to improve wound healing. [7-10] However, limitations to this technology included the requirement for frequent dosing due to the short half-life of growth factors in tissue. A longer lasting expression of growth factors can be feasibly achieved with

DNA plasmid expression vectors, where a cell produces its own growth factors encoded by transfected DNA plasmids.

Previous methods of plasmid transfection *in vivo* faced significant barriers to clinical use for lack of transfection efficiency.[11-15] Injection of naked DNA plasmid requires prohibitively high levels of plasmid due to extremely low cell uptake efficiency. At such high concentrations, the plasmid vectors themselves interfere with wound healing.[15] The gene gun suffers the disadvantage of producing variable results from the same gun, often requiring repeated administration to achieve a desired result.[12, 13] Adenovirus has been found to have high transfection efficiency, but at the same time it is currently under scrutiny for its potential association with the serious medical risk of producing the systemic inflammatory response syndrome.[16]

Electroporation (EP) has been used since the 1980's as an efficient means of plasmid transfection *in vitro*.[17] An electric field is applied to the cell, which is believed to create a transmembrane voltage potential that causes a disruption in the continuity of the plasma membrane.[18] This transiently allows the passage of charged macromolecules such as negatively charged DNA plasmids.[19] It has been shown that an electrophoretic effect created by the applied electric field enhances the migration of DNA into cells.[20]

More recently, electroporation has been used *in vivo* to increase intracellular delivery of chemotherapeutics directly into tumors, and to enhance transdermal drug delivery.[18] Improvements in transfection of liver[21], muscle[22], and tumor[23] *in vivo* have all been recently demonstrated using electroporation. We and others have shown that EP facilitates delivery of

DNA plasmid expression vectors to cutaneous tissue.[24-27] We have demonstrated the efficacy of electroporation-assisted transfection into abnormal, injured cutaneous tissue and its potential for improving wound healing in a diabetic mouse model. [1, 28]

In the current study we extended our prior work by using electroporation as an *in vivo* method of plasmid transfection in a septic rat wound healing model. First we induced sepsis in rats via a partial cecal ligation procedure, and showed that wound healing was impaired by this procedure. Then we used a luciferase DNA plasmid expression vector in wounded rats to assess the efficacy of electroporation-assisted naked plasmid transfection compared to transfection without electroporation. We then used the septic rat model of wound healing to determine the effect of electroporation and KGF DNA plasmid transfection on the rate of cutaneous wound closure and histologic quality of healing in the septic rat model. We also demonstrated a tissue-specific effect of electroporation-enhanced DNA plasmid delivery.

Methods

Animals

Female 6-8 week old Sprague Dawley rats were obtained from Harlan, Inc (Indianapolis, IN). All procedures were approved by the Johns Hopkins University Animal Care and Use Committee.

Rat Sepsis Model

For wound healing experiments, an LD20 Sprague Dawley rat sepsis model of wound healing with partial cecal ligation was utilized.[29] The rats were anesthetized with an intramuscular

injection of 50 mg/kg Ketamine. A 4 cm long mid-line incision was created in the abdomen. The cecum was mobilized from the ascending colon. A small amount of feces was pushed into the distal portion of the cecum -- enough to be trapped after ligation but not an excessive amount that would create distention and pressure. The ligation was done with Silk 2-0 at mid-portion without ligating the blood vessels. No puncture of the cecum was performed, as we found in previous experiments that this causes excessive mortality (LD80). Such a high mortality rate makes finding a measurable difference in wound healing between groups difficult. Without puncture, we produced an LD20 model, which was appropriate for these studies. The abdominal wound was closed in two layers with Ethicon 4-0. Wounds were left undressed and animals were housed individually. For the luciferase experiments, a non-septic rat model was used.

Plasmids

The Plasmid gWIZ-Luc, containing a CMV promoter and luciferase transgene, was obtained from Gene Therapy Systems (San Diego, CA). Plasmid pCDNA3.1/KGF-1 was obtained from Invitrogen (Carlsbad, CA). Using gene specific PCR primers incorporating restriction enzyme sites for Pst1 and BamH1, KGF-1 was PCR amplified from pCDNA3.1/KGF and ligated into the corresponding restriction enzyme sites in gWIZ (Gene Therapy Systems, San Diego, CA). Plasmids were purified using an endotoxin free plasmid purification kit (Qiagen, Santa Clarita, CA) following culture in transformed DH-5α bacteria. Plasmids were stored at -70°C at a concentration of 2 mg/ml until use.

PCR

Conventional PCR was performed using a standard PCR kit from Invitrogen (Carlsbad, CA) and carried out according to manufactures instructions.

PCR primers:

OJW007: 5'AACTGCAGATGCACAAATGGAT 3'

OJW004: 5' TTAAGTTATTGCCATAGGAAG 3'

Wounding and Plasmid Administration

Animals were still under anesthesia from the cecal ligation surgery prior to cutaneous wounding, plasmid administration and EP. Rats in the luciferase experiments had not undergone cecal ligation therefore they were anesthetized with an intramuscular injection of 50 mg/kg Ketamine. Their dorsum was shaved and two, four or six symmetrical full thickness excisional wounds were created on their backs using an 8 mm punch biopsy instrument. Equal numbers of wounds were created on the left and on the right side. Plasmid DNA (50μg) in 50μl of phosphate buffered saline (PBS) was injected intradermally anterior and posterior to the wound site. The resulting skin blebs confirmed intradermal delivery of the plasmid and were marked with indelible ink. Wounds were left undressed and animals were housed individually.

Electroporation

Animals were electroporated at the site of injection within two minutes of plasmid administration, using a square wave electroporator (ECM 830, BTX Genetronics, San Diego, CA). A custom designed pin electrode, consisting of two 10mm rows of parallel needles separated by 5 mm was used to apply the electroporation voltage. Six square wave pulses were

administered, at an amplitude of 1800 volts, 100 μs duration, with a pulse interval of 125 ms. These EP parameters were identified as appropriate for cutaneous tissues in prior experiments. [27]

In vivo Luciferase Imaging

To assess luciferase expression with and without electroporation, animals were analysed using an *in vivo* luciferase imaging system. In these experiments, rats were wounded and injected with plasmid as previously described, but only some groups of animals were electroporated at each transfection site using six 100μs pulses of 1800V/cm, with an interval of 125ms, while the other group remained unelectroporated.

For the luciferase imaging, at time points after the initial transfection, animals were sedated with intraperitoneal injections of 0.025mL/g of Ketamine, and then injected intraperitoneally with 140μL of 15mg/mL of D-luciferin in PBS. After a conventional light photograph was taken, bioluminescent images were acquired using a cooled charged coupled device camera (IVIS, Xenogen, Alameda, CA). Luminescent images were taken 30 minutes following luciferin administration, during which time the light emission had been shown to be in a plateau phase. [27] Bioluminescent images were overlaid onto the conventional image of each animal, and the light emission, corrected for background luminescence, was calculated for each injection site using image analysis software (Living Image, Xenogen, Alameda, CA). The different spectral colors represent a linear scale of the intensity of luminescence, corresponding to the total number of photons emitted per second from a square cm of tissue. The color scale is standardized for all output images (Minimum: 15,000 photons/sec/cm^2, Maximum: 1,000,000 photons/sec/cm^2). (Figure 2.)

Wound Healing Measurements

The wound eschar was carefully removed and the unepithelialized wound border traced *in situ* onto clear acetate paper. Images were digitized at 600 dpi (Visioneer Paperport 6000, Visioneer, Fremont, CA) and wound areas were calculated using image analysis software based on NIH image (Scion Image, Frederick, MD). Areas were expressed as a pixel count.

Histologic Grading of Quality of Wound Healing

The quality of healing was assessed histologically using a point scale from 1-4. Wound tissue was excised after the animals were anesthetized with Ketamine as before. Tissue was fixed in 10% formalyn, embedded in paraffin, and 15 μm thick slices were stained with hematoxylin and eosin. The grading system was as follows: 1. Incomplete epithelialization with open areas. Inflammatory cells and hemorrhage present in subcutaneous tissue. 2. Thin, but complete epithelium. 3-5 cell thickness. Continuing subcutaneous inflammatory reaction. 3. Thicker epithelial coverings, complete with 5-10 cell thickness. Subcutaneous tissue well healed. 4. Reticulated epithelium, 10-15 cell thickness. Subcutaneous tissue well healed with dense scar. (Figure 8.) The histological grade of each wound was assigned by four blinded observers. The final score for each wound was the average score from all observers.

Statistical Analysis

Results were presented as means ± standard error of the mean (SEM). Differences in means between groups were analyzed for significance using Student's t-test or ANOVA as appropriate using SigmaStat (SYSTAT Software Inc., Point Richmond, CA).

Results

Effects of Partial Cecal Ligation on Wound Healing

To induce sepsis, partial cecal ligation was performed on experimental rats.

We found impairment of wound closure in the septic group. Average wound area was approximately 4.7 times larger on day 9 when comparing rats who underwent partial cecal ligation to control rats undergoing sham surgery (18901 +/- 1636 vs. 4044.5 +/- 357 pixels, respectively; $p<0.001$; Figure 1).

Luciferase Plasmid Expression Efficiency

We used the luciferase assay to determine if electroporation significantly enhanced luciferase plasmid transfection efficiency in wounded cutaneous tissue in the rat. Luciferase plasmid transfection with and without EP was compared. Rats were divided into two groups, consisting of 3 rats each. In each group six excisional wounds were created on the dorsum, three wounds on the left side and three on the right side. In both groups luciferase plasmid DNA was intradermally injected adjacent to all six wounds. DNA injection sites in the first group were not electroporated, whereas rats in the second group were electroporated at all six DNA injection sites. Comparing transfection with or without EP we observed that EP enhanced luciferase

expression 53-fold (140,000 +/- 58,000 vs. 7,520,000 +/- 970,000 photons/sec, $p<0.001$). (Figure 3.)

Single Versus Double Electroporation

The luciferase assay was also used to compare single electroporation with double electroporation (two applications of electroporation-assisted transfection separated by 24 hours). Double electroporation did not produce significantly different luciferase expression when compared to single electroporation. (Figure 4.)

Subcutaneous Versus Intradermal Injection

Next, we used the luciferase assay to determine whether intradermal (with bleb) or subcutaneous (no bleb) injection of luciferase plasmid produced higher luciferase expression. We found that subcutaneous injection produced minimal luciferase expression. In contrast, a robust expression was observed for intradermal injection, 63-fold higher than subcutaneous injection (3,230,000 +/- 810,000 vs. 52,000 +/- 17,000 photons/sec $p<0.0002$). (Figure 5.)

Localization of DNA Plasmid Expression

We next assessed whether the plasmid DNA expression vector remained in the cutaneous tissue or spread to the adjacent muscles of the carcass. We injected the luciferase plasmid intradermally in three places on the left dorsum of each rat with mirror image subcutaneous injections on the right. (Figure 6.) We imaged the intact rat as before, but then dissected off the cutaneous tissue and re-imaged the strips that had been transfected after inverting them. We also imaged the dorsal musculature as seen on Figure 6. A robust signal from the luciferase produced

by the DNA plasmid expression vector was seen localized to the skin after intradermal injection, and this expression remained limited to the cutaneous tissue. Minimal luciferase activity was occasionally observed on the areas transfected via subcutaneous injection, for both dorsal cutaneous and dorsal muscle views. We attribute the minimal signal to possible inadvertent intradermal injection during the subcutaneous infusion.

Wound Healing

We assessed the effects of electroporation on the rate of wound healing in the septic rat model. We had shown that wound healing is impaired in septic animals. (Figure 1.) We assessed the ability of the KGF-1 DNA expression vector to improve cutaneous wound healing in this model. Electroporation-enhanced KGF growth factor plasmid transfection was compared to KGF growth factor transfection without electroporation. Single EP transfection of a KGF plasmid vector improved wound healing as evidenced by an average of 60.0% smaller wound areas on Day 12 in the KGF+EP vs. KGF animals without electroporation (460 +/- 78 vs. 1149 +/- 260 pixels, $p<0.009$). (Figure 7.)

Histologic Grading of Quality of Wound Healing

In order to understand the histological effects of KGF with electroporation on wound healing, we microscopically examined wounds using the previously described grading system. We compared wound healing using KGF with EP versus KGF without EP. KGF with EP on average showed a 67% higher score than KGF without EP (3.0 +/- 0.3 vs 1.8 +/- 0.3, $p<0.05$). (Figure 9.)

Discussion

In our experiments we showed that wound healing was impaired by sepsis. We demonstrated that the expression of DNA plasmid vectors was enhanced by electroporation. We also established that electroporation-enhanced delivery of DNA plasmid expression vectors for KGF-1 significantly improved the rate and quality of wound healing in septic rats. We showed that, using skin-specific EP parameters, transfection of DNA plasmid was localized to cutaneous tissue and did not spread to adjacent muscle.

Impaired wound healing in septic individuals remains a major unsolved problem in trauma as well as other settings. [30, 31] Rico et al. [6] showed delayed reepithelialization and collagen synthesis in the wounds of septic, cutaneously wounded mice. They found that peripheral neutrophil count was elevated in the infected animals, while the number of neutrophils and macrophages at the wound site itself was reduced. Thornton et al. [29] also found that collagen synthesis is impaired for colon anastomotic wounds in a septic rat model.

The present studies confirm the impairment in cutaneous wound healing in the rat sepsis model. They also show that a plasmid DNA expression vector for KGF-1 delivered with electroporation can improve wound healing in this model. Both the quality of healing and the speed of closure of cutaneous wounds were improved.

Further studies using a luciferase vector confirmed that the transfection acted locally and did not spread into adjacent tissue. This is an important issue related to the potential clinical use of this modality. For clinical use it is desirable to have expression of a plasmid DNA expression vector

limited to the target tissue. Our system was found to demonstrate this type of localized expression. Localized expression reduces the risk that a growth factor or other products of the delivery system will have undesirable effects on tissue remote from the wound target.

There are several potential advantages of using EP for wound healing. With EP, the concentration of plasmid required to achieve effective transfection is much lower than with naked plasmid transfection. We have previously found that the high plasmid load required to achieve transfection with naked plasmids is itself detrimental to wound healing. [15] Also, the localized nature of electroporative therapy makes it amenable to the use of local anesthesia alone. In addition, no viral vector is used, avoiding the risk of local and/or systemic inflammatory response, which may impair wound healing. Furthermore, no foreign bodies are utilized, such as with the gene gun. EP also produces less variable results than the gene gun. [12, 13] Another potential advantage of EP is that tissue specific parameters can target therapy to relatively precise locations. This, for example, would allow selective transfection of skin but not muscle, or vice versa.[24] For other purposes muscle specific parameters could be used to transfect adjacent particular muscle. It may or may not be possible to identify EP parameters that would allow direct transfection of subcutaneous tissue or the granulation tissue of the wound itself. These approaches may have the potential to be even more effective than the present approach, and furthermore to extend the approach to improve healing beyond just cutaneous tissue. Finally, EP itself using skin parameters is not detrimental to healing [27], as supported by the improvement in both the rate and histologic quality of healing with KGF plasmid demonstrated in our experiment.

For the military or civilian trauma casualty this approach has great relevance. Abdominal trauma with perforation of bowel is often complicated by sepsis. The casualty must often heal multiple other injuries to the extremities, head, and abdominal wall while sepsis is being controlled. An approach that will improve wound healing in this setting has important potential value.

Using electroporation, the DNA plasmid product, in this case KGF-1, was able to influence the healing of the targeted wound. This has multiple implications for the use of electroporation in localized delivery of DNA plasmid expression vectors to wounded tissue, as the expression of any number of gene sequences can potentially be enhanced, including growth factors, hormones, developmental signaling peptides, and immunizing factors. An optimal approach may include a number of different expression products working together synergistically.

References

1. Marti G, Ferguson M, Wang J, et al. Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model. Gene Ther 2004; 11(24):1780-5.
2. Stamm J, Cooney R, Maish G, et al. Growth hormone does not attenuate the inhibitory effects of sepsis on wound healing. Wound Repair Regen 2000; 8(2):103-9.
3. Maish Gr, Shumate M, Ehrlich H, Cooney R. Tumor necrosis factor binding protein improves incisional wound healing in sepsis. J Surg Res 1998; 78(2):108-17.
4. Cooney R, Iocono J, Maish G, et al. Tumor necrosis factor mediates impaired wound healing in chronic abdominal sepsis. J Trauma 1997; 42(3):415-20.
5. Mertz P, Ovington L. Wound healing microbiology. Dermatol Clin. 1993; 11(4):739-47.
6. Rico R, Ripamonti R, Burns A, et al. The effect of sepsis on wound healing. J Surg Res 2002; 102(2):193-7.
7. Robson M, Phillips L, Lawrence W, et al. The safety and effect of topically applied recombinant basic fibroblast growth factor on the healing of chronic pressure sores. Ann Surg 1992; 216:401-6.
8. Steed D. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers. J Vasc Surg 1995; 21:71-8.
9. Robson M, Phillips L, Thomason A, et al. Recombinant human platelet-derived growth factor-BB for the treatment of chronic pressure. Ann Plast Surg 1992; 29:193-201.
10. Wolf S, Nicolai M, Dagenais G. Growth hormone treatment in catabolic states other than burns. Growth Horm IGF Res 1998; 8(Suppl B):117-9.
11. Sun L, Xu L, Chang H, et al. Transfection with aFGF cDNA improves wound healing. J Invest Dermatol 1997; 108(3):313-8.

12. Andree C, Swain W, Page C, et al. In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. Proc Natl Acad Sci U S A 1994; 91(25):12188-92.

13. Eming S, Whitsitt J, He L, et al. Particle-mediated gene transfer of PDGF isoforms promotes wound repair. J Invest Dermatol 1999; 112(3):297-302.

14. Wolff J, Malone R, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990; 247(4949 Pt 1):1465-8.

15. Byrnes C, Khan F, Nass P, et al. Success and limitations of a naked plasmid transfection protocol for keratinocyte growth factor-1 to enhance cutaneous wound healing. Wound Repair Regen 2001; 9(5):341-6.

16. Felgner P, Rhodes G. Gene therapeutics. Nature 1991; 349(6307):351-2.

17. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider P. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1982; 1(7):841-5.

18. Prausnitz M, Bose V, Langer R, Weaver J. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. Proc Natl Acad Sci U S A 1993; 90(22):10504-8.

19. Somiari S, Glasspool-Malone J, Drabick J, et al. Theory and in vivo application of electroporative gene delivery. Mol Ther 2000; 2(3):178-87.

20. Satkauskas S, Bureau M, Puc M, et al. Mechanisms of in Vivo DNA Electrotransfer: Respective Contributions of Cell Electropermeabilization and DNA Electrophoresis. Mol Ther 2002; 5(2):133-40.

21. Suzuki T, Shin B, Fujikura K, et al. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett 1998; 425(3):436-40.

22. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther 1999; 6(4):508-14.

23. Wells J, Li L, Sen A, et al. Electroporation-enhanced gene delivery in mammary tumors. Gene Ther 2000; 7(7):541-7.

24. Drabick J, Glasspool-Malone J, King A, Malone R. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 2001; 3(2):249-55.

25. Titomirov A, Sukharev S, Kistanova E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. Biochem Biophys Acta 1991; 1088(1):131-4.

26. Heller R, Schultz J, Lucas M, et al. Intradermal delivery of interleukin-12 plasmid DNA by in vivo electroporation. DNA Cell Biol 2001; 20(1):21-6.

27. Byrnes C, Malone R, Akhter N, et al. Electroporation Enhances Transfection Efficiency in Murine Cutaneous Wounds. Wound Repair Regen 2004; 12(4):397-403.

28. Ferguson M, Byrnes C, Sun L, et al. Wound Healing Enhancement: Electroporation to Address a Classic Problem of Military Medicine. World J Surg In press.

29. Thornton F, Ahrendt G, Schaffer M, et al. Sepsis Impairs Anastomotic Collagen Gene Expression and Synthesis: A Possible Role for Nitric Oxide. Journal of Surgical Research 1997; 69(1):81-86.

30. Clark M, Plank L, Hill G. Wound Healing Associated with Severe Surgical Illness. World J Surg 2000; 24(6):648-654.

31. Williams D, Harding K. Healing responses of skin and muscle in critical illness. Crit Care Med 2003; 31(8):S547-57.

Figure Legends

Figure 1. Comparison of the wound areas on day 9 in septic (partial cecal ligation) vs. non-septic animals. Average wound area (pixels) +/- SEM is shown. Average wound area of septic rats who underwent partial cecal ligation were approximately 4.7 times larger on day 9 when comparing rats to control rats undergoing sham surgery ($p<0.001$).

Figure 2. Example of computerized luciferase graphical output image from IVIS imaging system taken from a luciferase experiment. EP increased the luminosity produced.

Figure 3. Comparison of average luciferase expression on day 5 with and without EP bilaterally. Average luciferase activity (photons/s) +/- SEM is shown. Transfection with EP showed a 53-fold increase in luciferase expression ($p<0.001$).

Figure 4. Comparison of luciferase expression for single vs. double EP. W=Wound, P=Plasmid, 'Single EP' = W+P+EP, 'Double EP' = W+2(P+EP). Average luciferase activity (photons/s) +/- SEM is shown. Double EP did not produce significantly more luciferase expression than single EP.

Figure 5. Luciferase expression of intradermal vs. subcutaneous transfection. Average luciferase activity (photons/s) +/- SEM is shown. Intradermal transfection produced 63-fold higher luciferase expression than subcutaneous transfection ($p<0.0002$). With skin-specific EP parameters the transfection was localized to cutaneous tissue.

Figure 6. Dorsal, ventral and subdermal views of luciferase expression for intradermal (left dorsum) vs. subcutaneous (right dorsum) transfection. Minimal luciferase activity was seen for subcutaneous transfection, and for both types of transfection minimal luciferase activity was seen from the underlying muscle.

Figure 7. Comparison of wound areas for KGF+EP vs. KGF. Average wound area (pixels) +/- SEM is shown. Transfection with EP produced 60% smaller wound areas by day 12 ($p<0.009$)

Figure 8. Histologic Grading Scale.

Figure 9. Mean histological grade for KGF+EP vs. KGF. Average histological grade (points) +/- SEM is shown. Transfection with EP produced a 67% higher histological grade ($p<0.05$).

Figures

Figure 1.

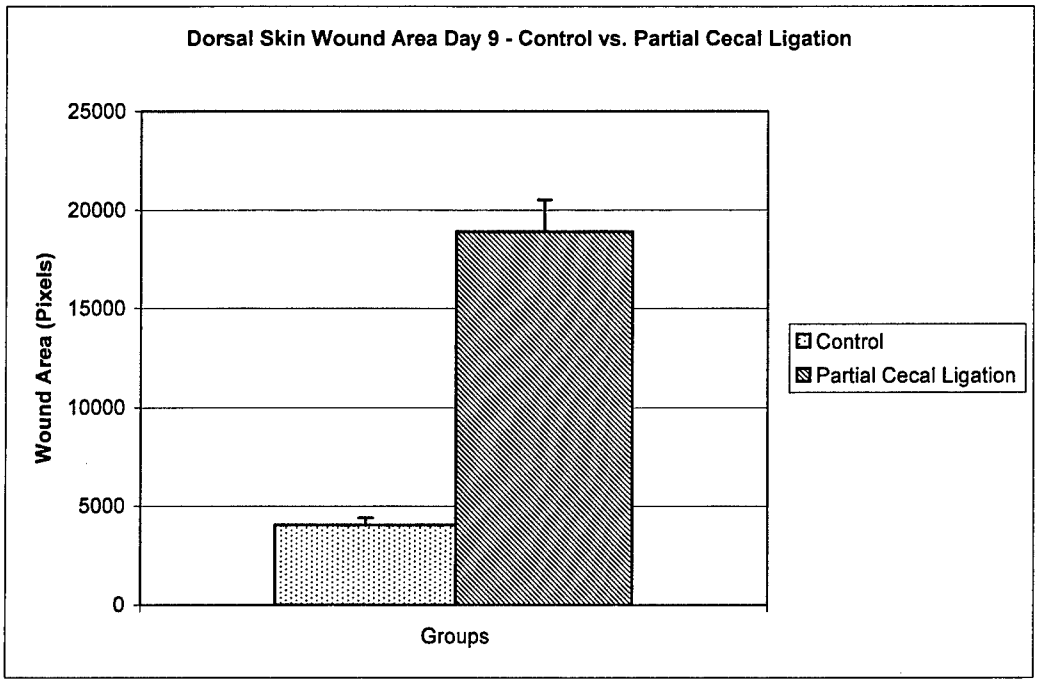
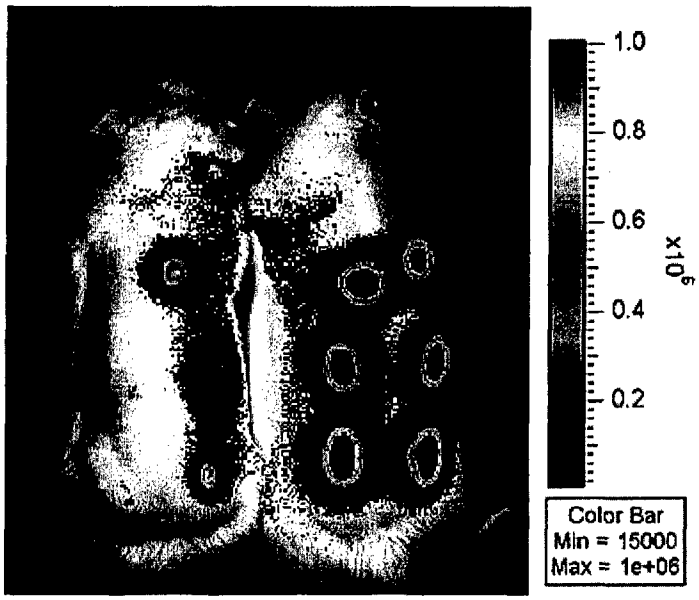
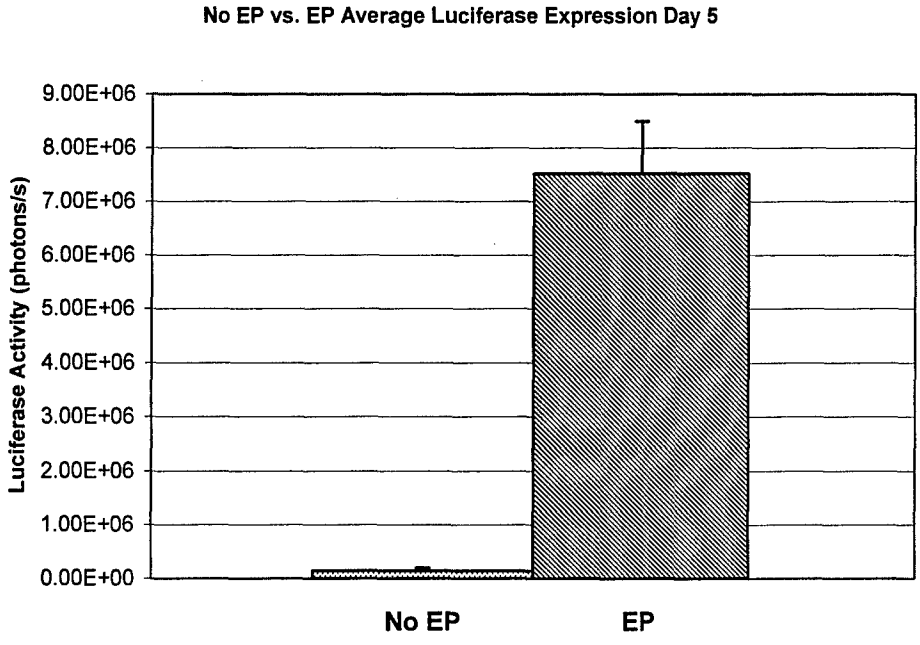


Figure 2.



Plasmid Plasmid+EP

Figure 3.


No EP vs. EP Average Luciferase Expression Day 5
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8.00E+06
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
No EP
EP

Figure 4.

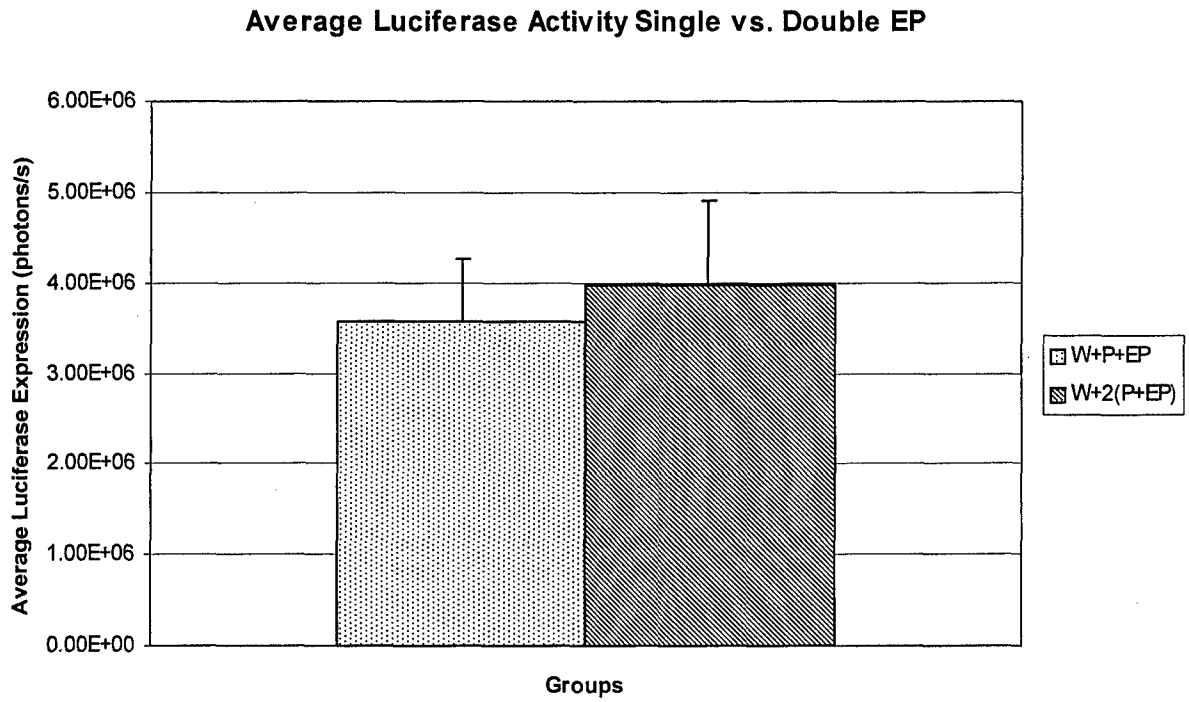
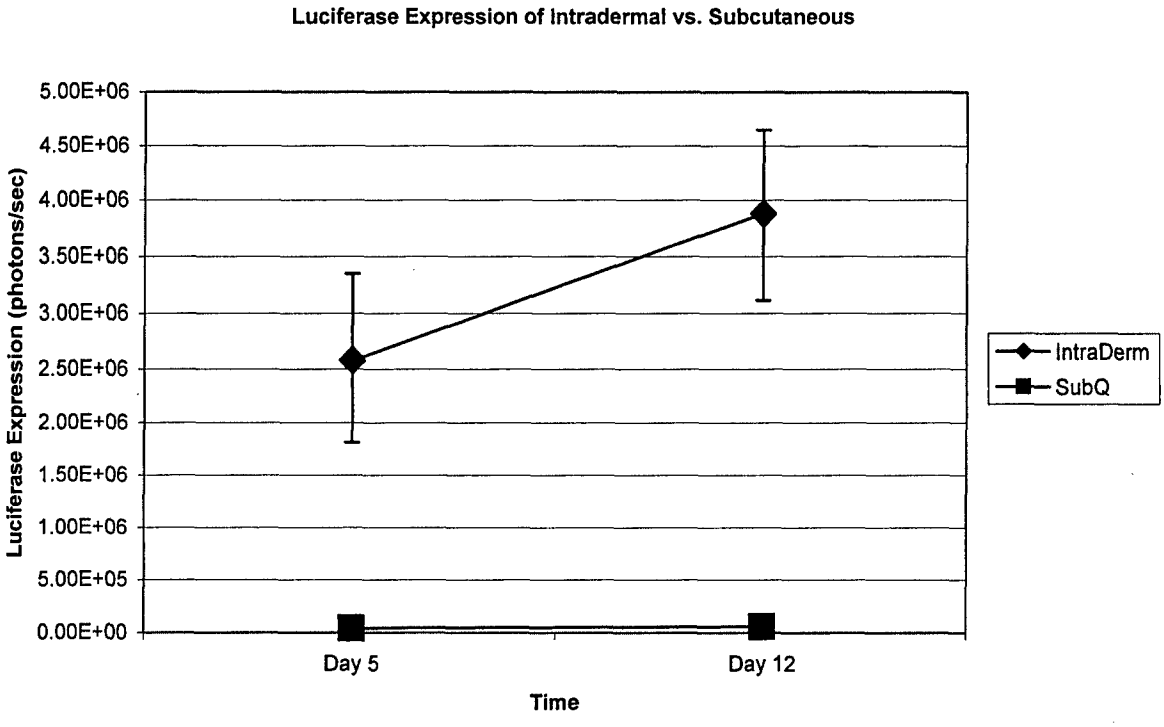

Average Luciferase Activity Single vs. Double EP
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
Average Luciferase Expression (photons/s)
Groups
W+P+EP
W+2(P+EP)

Figure 5.


Luciferase Expression of Intradermal vs. Subcutaneous
Luciferase Expression (photons/sec)
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2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Day 5
Day 12
Time
IntraDerm
SubQ

Figure 6.

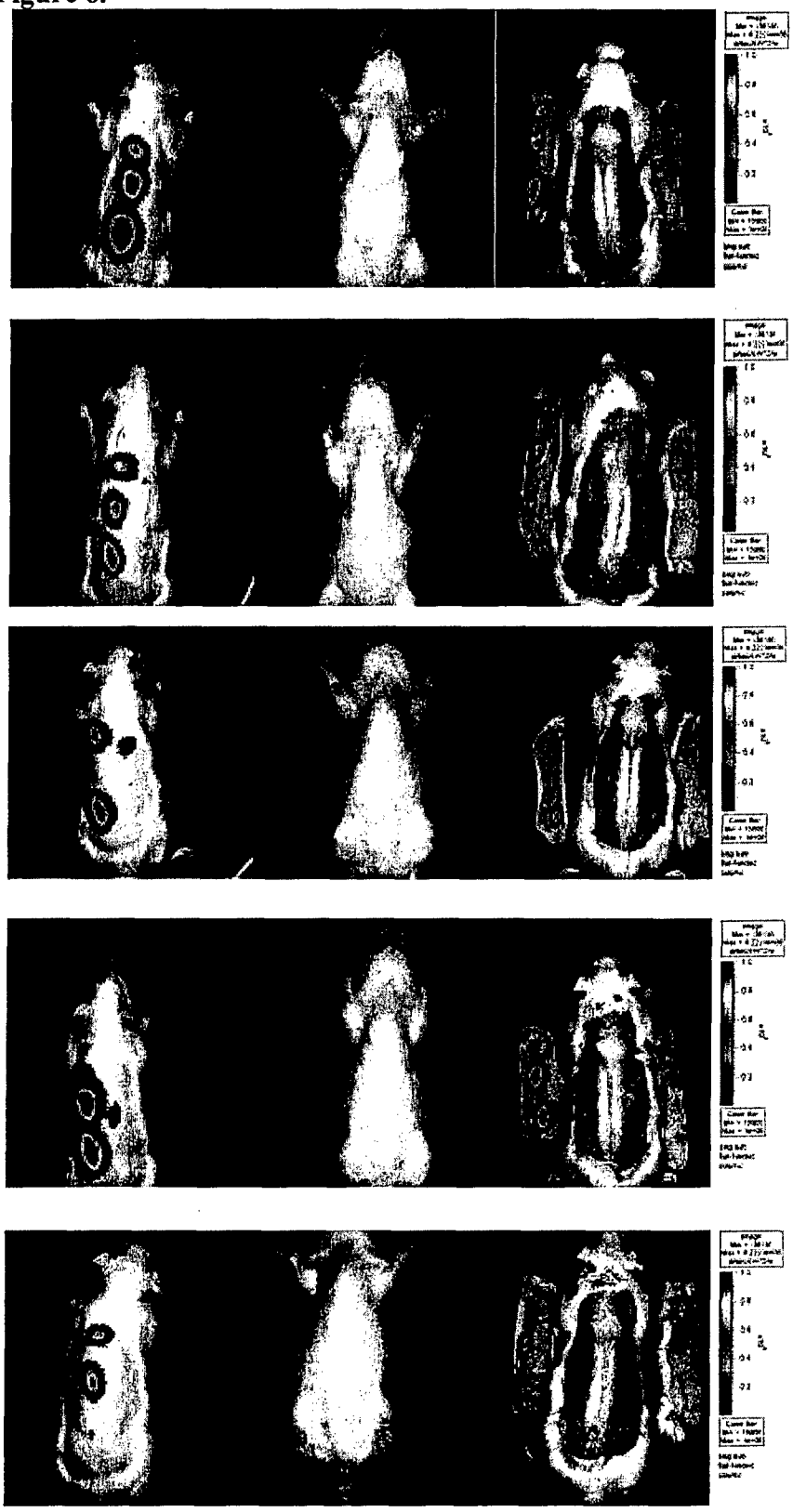


Figure 7.

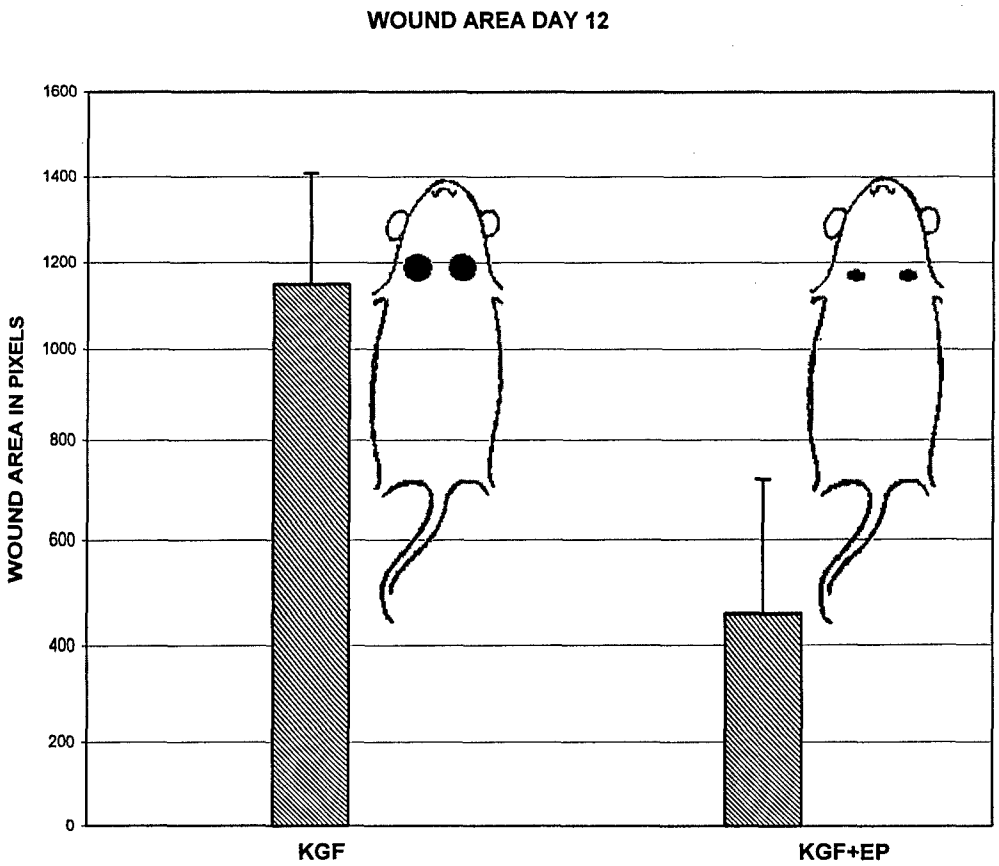
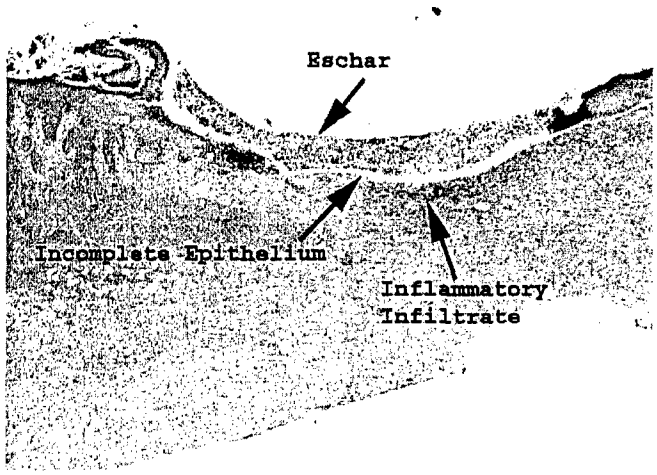
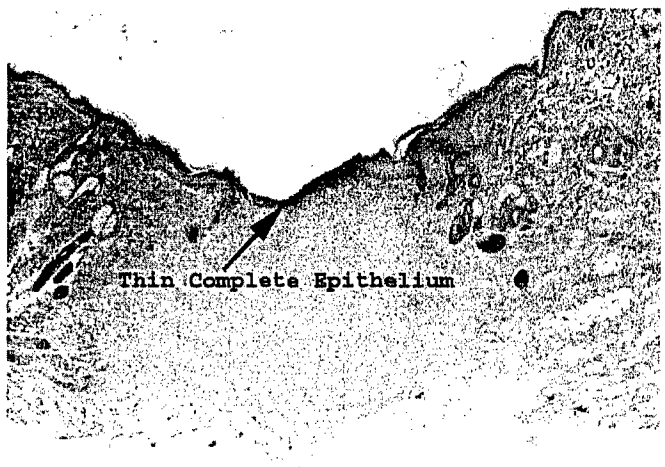

WOUND AREA DAY 12
WOUND AREA IN PIXELS
1600
1400
1200
1000
800
600
400
200
0
KGF
KGF+EP

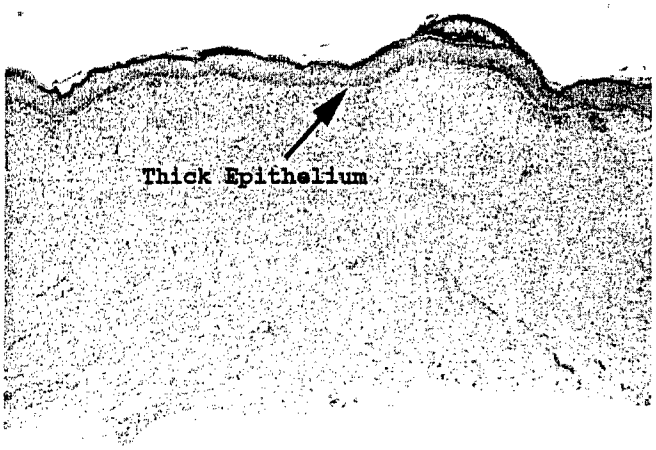
Figure 8.



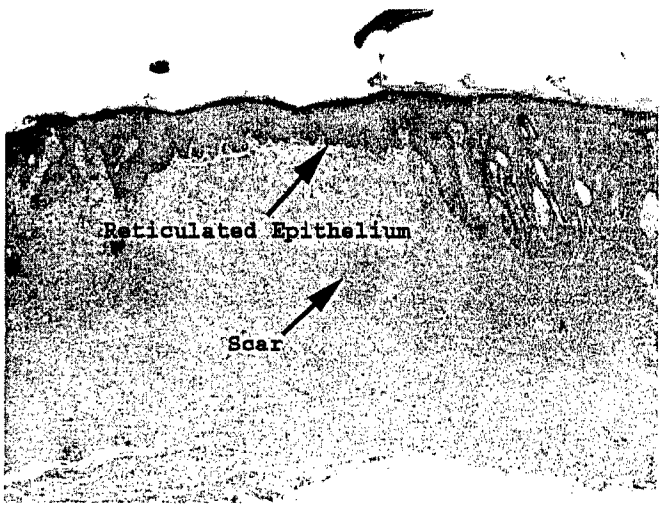
Grade 1



Grade 2

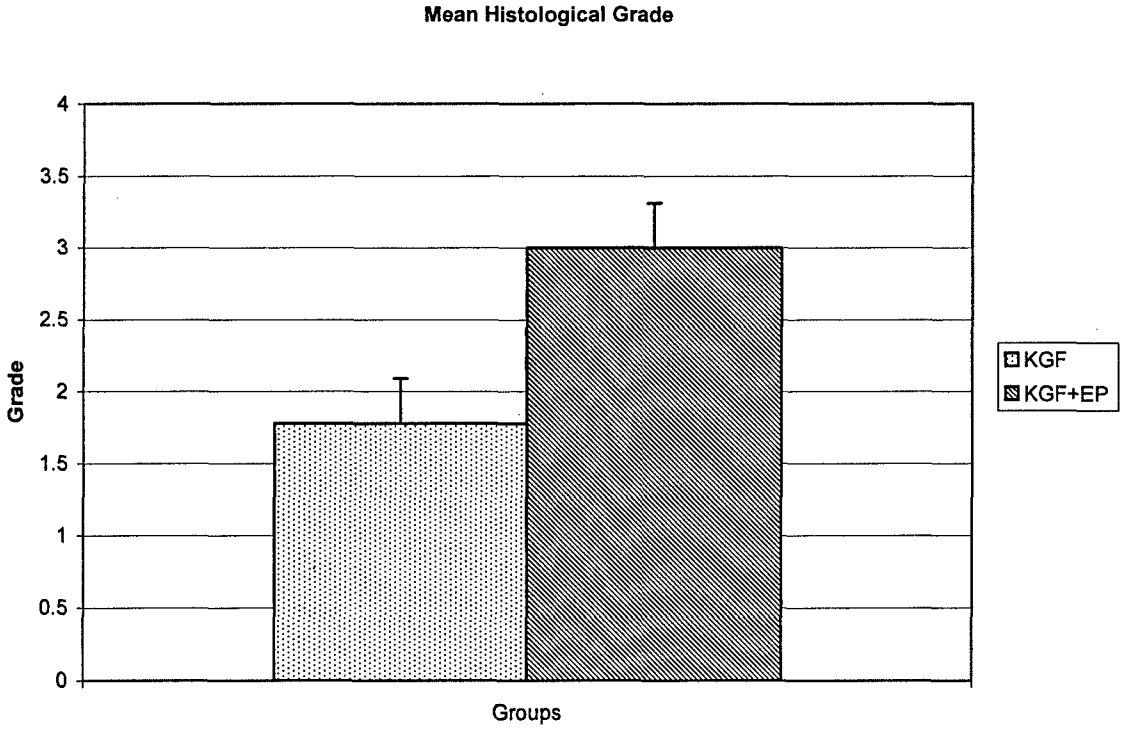


Grade 3



Grade 4

Figure 9.


Mean Histological Grade
4
3.5
3
2.5
2
1.5
1
0.5
0
Grade
Groups
KGF
KGF+EP